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Characterization of choline uptake in *Trypanosoma brucei* and involvement of a mitochondrial carrier in resistance to choline analogs

PhD Thesis submitted by

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Abstract

Choline is an essential nutrient for eukaryotic cells, where it is used as precursor for the synthesis of choline-containing phospholipids, such as phosphatidylcholine (PC). Our experiments showed – for the first time – that *Trypanosoma brucei*, the causative agent of human African sleeping sickness, is able to take up choline from the culture medium to use for PC synthesis, indicating that trypanosomes express a transporter for choline at the plasma membrane. Further characterization in procyclic and bloodstream forms revealed that choline uptake is saturable and can be inhibited by HC-3, a known inhibitor of choline uptake in mammalian cells.

To obtain additional insights on choline uptake and metabolism, we investigated the effects of choline-analogs that were previously shown to be toxic for *T. brucei* parasites in culture. Interestingly, we found that all analogs tested effectively inhibited choline uptake into both bloodstream and procyclic form parasites. Subsequently, selected compounds were used to search for possible candidate genes encoding choline transporters in *T. brucei*, using an RNAi library in bloodstream forms. We identified a protein belonging to the mitochondrial carrier family, previously annotated as TbMCP14, as prime candidate. Down-regulation of TbMCP14 by RNAi prevented drug-induced loss of mitochondrial membrane potential and conferred 8-fold resistance of *T. brucei* bloodstream forms to choline analogs. Conversely, over-expression of the carrier increased parasite susceptibility more than 13-fold. However, subsequent experiments demonstrated that TbMCP14 was not involved in metabolism of choline. Instead, growth curves in glucose-depleted medium using RNAi or knock-out parasites suggested that TbMCP14 is involved in metabolism of amino acids for energy production. Together, our data demonstrate that the identified member of the mitochondrial carrier family is involved in drug uptake into the mitochondrion and has a vital function in energy production in *T. brucei*.

Preface

Trypanosomes are extraordinary organisms. Several features about their (structural) biology and biochemistry attracted my curiosity, such as the fascinating structure and dynamics of the kinetoplast (see [1] for a stimulating review on this topic). By accident, the mitochondrion of *Trypanosoma brucei* became a major field of interest during my thesis when I discovered that a mitochondrial carrier protein mediated the action of trypanocidal choline analogs, allowing me to dive into mitochondrial pathways involved in energy metabolism.

1. Introduction

1.1 Heavy burden for African countries

African Trypanosomiases include human and animal diseases that are caused by trypanosomes. The animal form of the disease is called "Nagana", which in Zulu means "powerless" or "low in spirit", and is caused by *T. congolense, T. vivax* or *T. brucei*. The infection causes mild symptoms in wild animals while domesticated animals often suffer from a more aggressive form of Nagana, which is often fatal if not treated. Human African Trypanosomiasis (HAT) is known as sleeping sickness and occurs in two different forms: the chronic form is caused by *T. brucei gambiense* and is distributed in Northern and Western Africa. It is responsible for 90% of cases of sleeping sickness [2]. The acute form is caused by *T. brucei rhodesiense*, prevalent in Southern and Eastern Africa and being responsible for 10% of cases of sleeping sickness [2]. The distribution of HAT corresponds to the range of the insect vector, the tsetse fly, and includes 36 countries in sub-Saharan Africa, with approximately 70 million people in a surface of 1.55 million km^2 being at moderate to very high risk of contracting HAT [3].

Together, sleeping sickness and Nagana represent a very heavy burden to the development of rural areas of Africa. The human disease reduces labor resources and the animal related disease deprives the human population from meat, milk, and draught animal power. The Food and Agriculture Organization (FAO) estimates that Africa loses annually an equivalent of US$ 1.5 billion in income from agriculture as a result of African Trypanosomiases [4]. No vaccines are available against HAT, primarily because *T. brucei* bloodstream forms are elegantly elusive to recognition by the immune system by constantly changing the expression of surface proteins (see Chapter 1.5).

1.2 Life cycle of T. brucei

T. brucei are dixenous parasites, i.e. parasites having with a life cycle in two different host species. First, the tsetse fly from genus *Glossina ssp*. and invertebrate host of *T. brucei*, takes a blood meal from an infected mammalian ingesting a tsetse-infective form, called stumpy because of their morphology. Stumpy forms, which are not proliferative, starts differentiation into procyclic forms inside the midgut of the fly in order to guarantee their survival (Figure 1). This is the establishment stage of tsetse infection, in which the procyclic forms escape from digestion and proliferate [5,6]. When conditions are favorable, procyclic forms migrate to the salivary glands of a tsetse and undergo a second differentiation into epimastigotes, a proliferative stage that precedes the mammalian-

infective form called metacyclic. Metacyclics are non-proliferative, and morphologically different from procyclic forms and epimastigotes. During a second blood meal, metacyclic forms are inoculated into a mammalian host and develop into another blood stage, called long slender form [7] (Figure 1). Long slender parasites are replicative forms able to express a pool of variant surface glycoproteins.

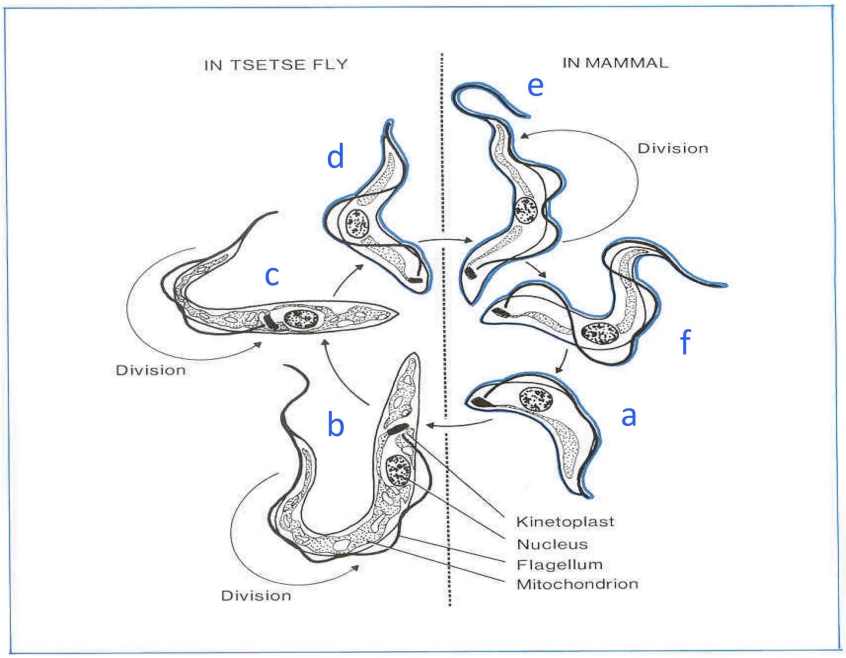

Figure 1. *T. brucei* life cycle. The cycle starts when tsetse-infective 'short' stumpy forms (a) are ingested by the fly and differentiates into procyclic forms (b). Inside the fly's midgut, the procyclic forms continue differentiation into epimastigotes (c) and to metacyclic (d) forms. When a new mammalian is bitten, the metacyclic forms enter in bloodstream and differentiates into the replicative 'long' slender forms (e), which differentiates into an intermediate stage (f) before becoming 'short' stumpy again (a) [6]. The blue line surrounding the cell body represents the VSG coat. Notice the mitochondrion development from f to b. Copied on 03.03.14, 08:30 am from: http://www.ilri.org/InfoServ/Webpub/fulldocs/Ilrad90/Trypano.htm .

1.3 Treatment of Nagana and sleeping sickness

Treatment of Nagana is commonly based on carcinogenic drugs like ethidium bromide and isometamidium chloride, which can compromise the meat used for human consumption if not carefully administrated. In addition, suramin and diminazene aceturate, both with considerable toxicity to cattle, are used for treatment [8]. There are five approved drugs to treat HAT [9]. Pentamidine and suramin are used in a first stage when parasites are still present in the hemolymphatic tissue. The second stage is characterized by the invasion of parasites into the central nervous system (CNS) and is treated with melarsoprol or combinations of nifurtimox and eflornithine [6]. Melarsoprol, the single drug against the acute and the chronic forms of sleeping sickness, is the

most toxic of all causing encephalopathies in 5% of cases. Moreover, there are well-known examples of cross-resistance in the field [10,11] demonstrating that development of resistant strains is a real threat. Hence, additional efforts to optimize the treatment of sleeping sickness and to develop new drugs are needed. However, despite new research technologies, development of novel and efficient drugs is not an easy task. To illustrate this point, I'm going to describe some inspiring stories about how the currently approved anti-kinetoplastid drugs were discovered.

1.4 Discovery of trypanocidal drugs

From 1896 to 1906 Africa faced a devastating epidemic of sleeping sickness that was responsible for 300'000-500'000 deaths in Congo, Uganda and Kenya [12]. This terrible outcome led various colonial administrations to ask scientists to develop a cure for sleeping sickness. At that time the field of medicinal chemistry, i.e. identification, synthesis and development of therapeutic use, was only emerging and used for the first time to seek for a new compound against sleeping sickness [13]. The development of the first anti-kinetoplastid took advantage of the well-advanced dyestuff market in Germany and the talent of a visionary scientist, Paul Ehrlich, who was the first to use dyes in the development of chemotherapies [13]. In 1901, Ehrlich tested more than 100 synthetic dyes against *T. equinum*, causative of 'mal de caderas' in equids, and *T. b. brucei*, resulting in the selection of only one reactive dye, named Nagana red (Figure 2) [13]. The curative effects of Nagana red in *T. equinum*-infected mice was not satisfactory, which made Ehrlich hypothesize that this could be explained by the low solubility of the compound. A few years later, a derivative of Nagana red was synthesized by Ludwig Benda and Cassella Farbwerke and was named Trypan red [13]. The new compound was found to cure *T. equinum*-infected mice, but was not active against other species of *Trypanosoma*. In 1906, the German pharmaceutical company Bayer took over the task to find a new trypanocidal compound and identified a blue dye named as Trypan blue (Figure 2) to be very toxic against all species of trypanosomes [14,15]. However, it was not suitable to treat humans because of its side effect: the skin of patients was becoming blue. Only in 1917, after screening between 1000 naphtalene ureas a very promising compound was selected and tested, showing to be effective in eliminating trypanosomes in animal and humans [16]. The drug was re-named suramin (Figure 2) and is still in use to treat the first stage of *T. brucei gambiense* infections until today.

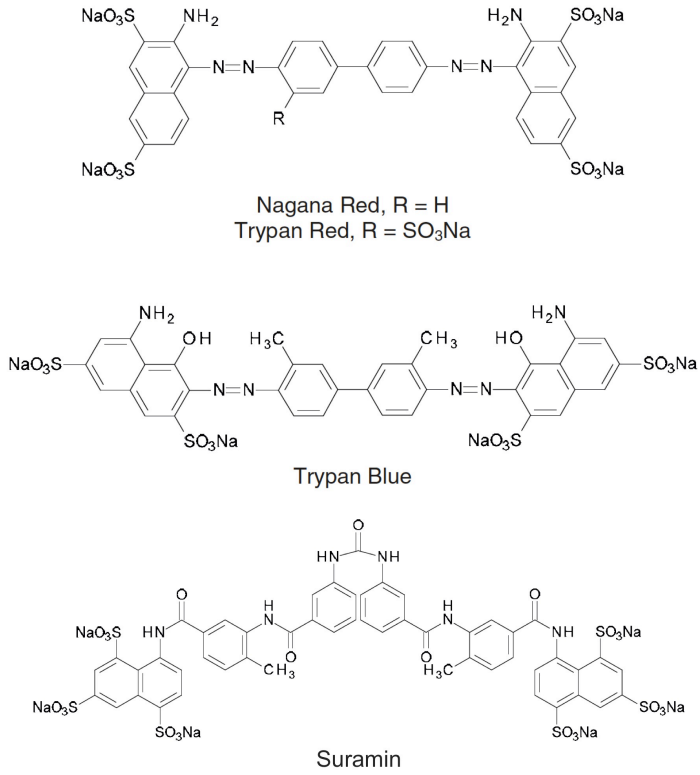

Figure 2. Chemical structures of sulphated naphthylamine derivatives with trypanocidal activities. Suramin is the oldest approved drug against HAT, it still in use to treat the first stage of the disease. Adapted figure reproduced from Steverding 2010 [13].

Arsenicals were first mentioned to exert curative properties by missionary David Livingston who recommended Fowler's Solution (a solution of 1% potassium arsenite) to treat sleeping sickness [13]. In 1902, Charles Louis Alphonse Laveran, a French physician who discovered the malaria parasite, and the French zoologist Felix Mesnil reported that sodium arsenite was effective in clearing trypanosomes from infected laboratory animals despite the fact that few days later parasitaemia increased again and caused death of the experimental animals [17]. Few other arsenicals were tested during subsequent years, one of them being atoxyl (Figure 3) [18], initially believed to have marginal toxicity to humans, but later proved wrong by Robert Koch [13]. A promising derivative of atoxyl, arsenophenylglycine (Figure 3), was tested in humans in 1907 and was shown to be effective and safe, except in a low number of patients who developed a strong hypersensitive reaction [13,19]. In the same period, Paul Ehrlich obtained another analog of atoxyl as an attempt to improve chemotherapeutic properties to treat sleeping sickness but this time, there was no trypanocidal activity. However, a short time later, Ehrlich and his collaborator, Satachito Hata, showed that the same atoxyl analog was effective against syphilis and it became the first reliable drug against this disease [19]. The drug was marketed under the name of Salvarsan "the arsenic that

saves". In 1919, two American scientists synthesized tryparsamide (Figure 3), a derivative of atoxyl and the first effective drug against the second stage of sleeping sickness [20]. In spite of toxic effects on optical nerves of patients, tryparsamide was used until 1960s [13]. It was 1938 when the Swiss pathologist, microbiologist and chemist Ernst Friedheim synthesized melarsen, a melamide derivative of atoxyl, which later was combined with dimercaprol, an antidote used against poisoning by arsenic gas, to generate melarsoprol (Figure 3) [13,21]. The main advantage of melarsoprol was that it didn't affect the optic nerve, however, it caused reactive encephalopathies in 5% of patients, with 1-5% mortality [6]. Melarsoprol is still the only effective drug to treat the second stage of *T. brucei rhodesiense* sleeping sickness.

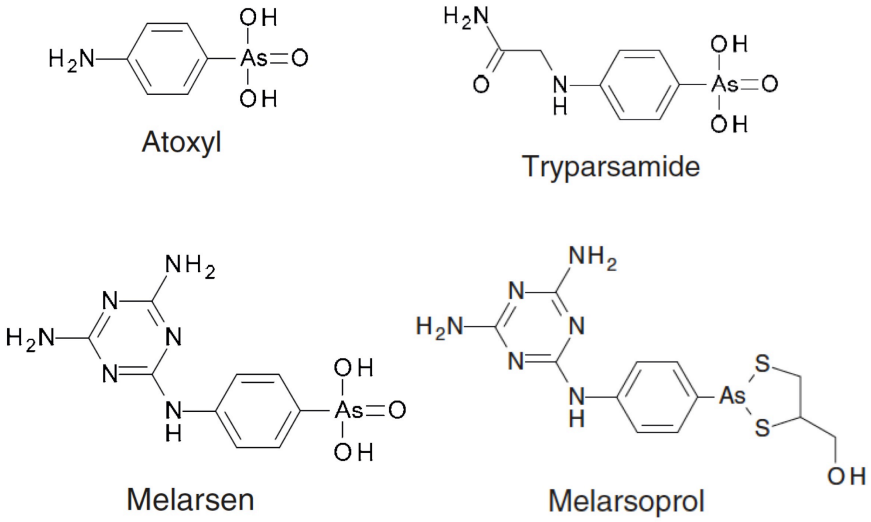

Figure 3. Chemical structures of arsenicals with trypanocidal activity. Only melarsoprol is currently used in the treatment of second stage of HAT. Adapted figure reproduced from Steverding 2010 [13].

As early as 1929, an innovative method to cultivate trypanosomes for short times led to the discovery that this parasite uses large amounts of glucose for normal growth [22]. Additionally, trypanosome-infected animals treated with insulin were shown to live longer and showed reduced parasitaemia [23]. As a result of this observation, a search for compounds able to reduce glycaemia led to the discovery of the trypanocidal effect of a diamidine, called synthalin (Figure 4) [13,24]. However, the effect against trypanosomes was later shown to be due to the toxic effect of synthalin itself and not because of the hypoglycemic action [25]. As a result, a large number of diamidines was synthesized and tested against trypanosomes, leading to the discovery of the highly trypanocidal compounds pentamidine and stilbamidine (Figure 4) [26,27]. This improved action against trypanosomes was, in part, due to the replacement of the polymethylene group, present in synthalin, by two phenyl groups (Figure 4) [13]. Stilbamidine displayed neurotoxic effect in humans and was

discontinued, whereas pentamidine is still used against the first stage of *T. b. gambiense* sleeping sickness.

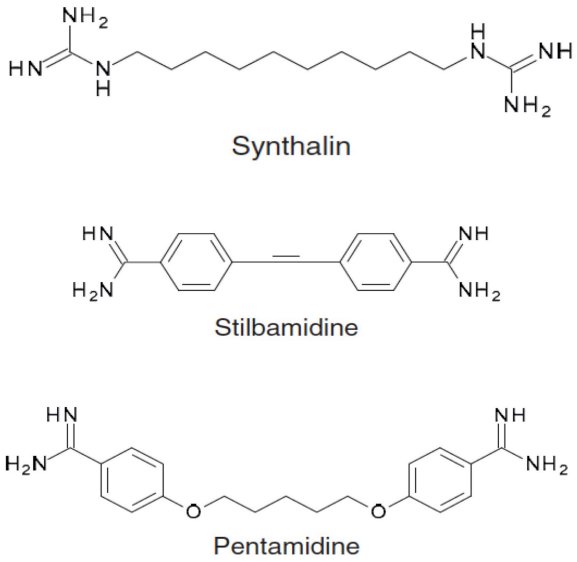

Figure 4. Chemical structures of diamidines with trypanocidal activity. Adapted figure reproduced from Steverding 2010 [13].

Eflornithine (Figure 5) was initially developed in the 1970s as a potential anti-cancer drug [28,29]. Only later it was found to cure *T. b. brucei* infections in murine models without any apparent side effects [30]. Eflornithine is known to irreversibly inhibit the activity of ornithine decarboxylase [29], the first enzyme involved in polyamine biosynthesis. This inhibition causes depletion of putrecine and spermidine, leading to reduced levels of trypanothione, an essential antioxidant in trypanosomes [31]. In 1990, eflornithine was approved for the treatment of HAT caused by *T. b. gambiense* and is currently the only treatment available for melarsoprol-refractory sleeping sickness. Interestingly, it has been shown that efflornithine is not active against *T. b. rhodesiense* due to the rapid turnover of ornithine decarboxylase in this sub-species, with a half-time of 4.3 h compared to 18-19 h in *T. b. gambiense* [32]. In 2009, the combination of eflornithine and nifurtimox, a trypanosomal drug initially used only against *T. cruzi*, was approved to treat the second stage of *T. b. gambiense* sleeping sickness [33]. This combination therapy resulted in a cheaper, safer and easier treatment of sleeping sickness.

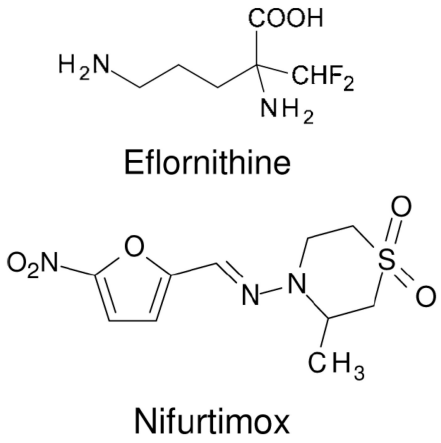

Figure 5. Chemical structures of eflornithine and nifurtimox, both used as a combination therapy against second stage of *T. b. gambiense* sleeping sickness. Adapted figure reproduced from Steverding 2010 [13].

1.5 Antigenic variation, the reason for chemotherapy

By definition, antigenic variations are changes in antigenic identity occurring more frequently than random mutation rates [34]. The concept of antigenic variation in *Trypanosoma* research was better accepted after the practical observation of waves of parasitaemia during *T. brucei* infections published in 1910 [35]. This observation was only possible after the development of better methods to count parasites and permitted the link between waves of parasitaemia and waves of fever that occur during sleeping sickness [5].

As an extracellular parasite, *T. brucei* is unable to hide from the mammalian immune system. As a result of this evolutionary pressure, parasites have developed the keen strategy of antigenic variation to escape from death by the immune response. This process is based on changes in the parasite's surface coat, which constitutes with >95% of a particular type of glycoprotein, the variant surface glycoprotein (VSG). A single type of VSG is expressed at a time from a repertoire of an estimated 1000 genes [36]. The active expression site of VSG is called bloodstream expression site (BES) or metacyclic expression site (MES), depending on the parasite stage the activation has occurred. The sites are present in telomeric regions of chromosomes. In the case of BES, there are an estimated 20-40 repeats in the genome. Each one produces a single polycistronic transcription unit of dozens of kilobases [34], which also contains several other genes named expression-site-associated genes (ESAGs) [36]. At any given time only one expression site is activated and only one VSG is transcribed. Therefore, trypanosomes have two possible mechanisms to change VSG expression. They can either replace the VSG gene within the active expression site or use a different expression site to produce a different VSG molecule [34]. Thus, the variety of possible antigens expressed in trypanosomal surface is enormous and is hard for the immune system to keep up mounting new efficient response against

trypanosomal antigens in the course of infection. *T. brucei* takes much shorter time to change its pool of antigens than the immune system to mount and exert a specific response against them. As a result, parasites that still expressing previous types of antigens are killed by the host defense but a minority of parasites expressing a new type of antigen will replicate without being recognized by the immune system (Figure 6).

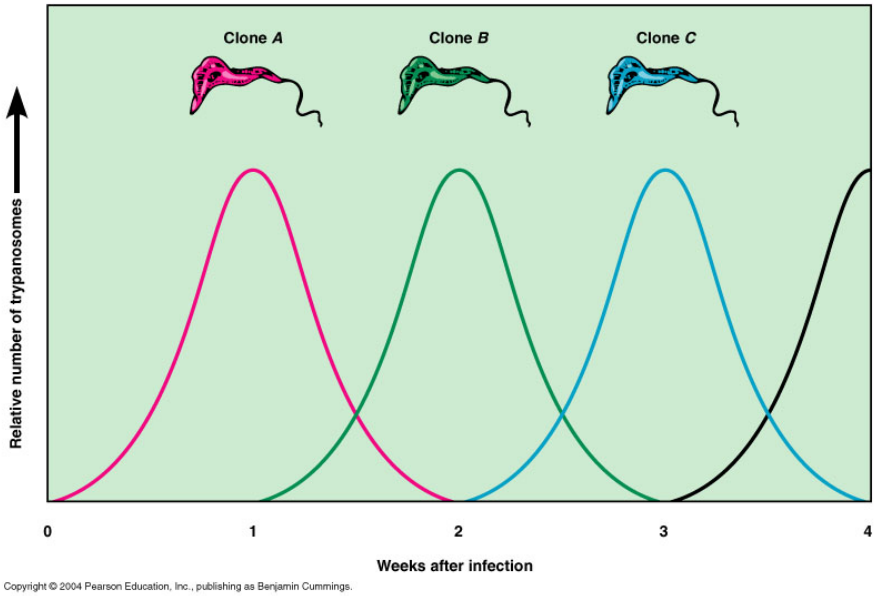

Figure 6. Waves of parasitaemia caused by antigenic variation. By the time the immune system of the mammalian host mounted an efficient response against *T. brucei*, some parasites are expressing a different type of surface antigen and are able to grow without interference until a new immune response is mounted. Downloaded from [37].

1.6 Trypanosomatidae diversity

According to a recent review [38], the identification of trypanosomatids in insects was documented for the first time in 1851 by Burnett [39], and the first genera, *Leptomonas* and *Herpetomonas*, were established in 1880 by Kent [40]. It was 1895 when the Scottish pathologist and microbiologist Sir David Bruce described *T. brucei* as the causative of cattle Nagana demonstrating the veterinary relevance of trypanosomatids [12], leading to the subsequent discovery of the agent of sleeping sickness in 1909 [41]. In 1903, another Scottish pathologist, William Leishman, reported a case of a soldier who served at Dum Dum, in the British India, and died in a state of gross emancipation and enlargement of the spleen [42,43]. In this case, Leishman could detect unknown parasites in slide preparations of spleen taken *post mortem*. In the same year, the physiologist Charles Donovan found the same parasites in several patients with enlarged spleen and the name *Leishmania donovani* was then suggested [44]. In 1904, *L. donovani* was accepted by most scientists as a new species of trypanosomatid and found to be the causative agent of kala azar, an intermittent fever with emancipation and enlargement of the spleen [45]. But only decades later, the sandfly was

unequivocally identified as the vector for kala azar [46]. A third pathogenic trypanosomatid was found in late 1907, when the Brazilian physician Carlos Chagas was coordinating prophylactic anti-malarian campaigns in Minas Gerais and heard about a blood-sucking 'barber bug', because of its predilection for biting victim's face. Chagas found a new species of trypanosome in the hindgut of the bug and, soon afterwards, the same parasite was found in the blood of a small girl as well as her cat's blood [47,48]. In subsequent analyses, Chagas succeeded in reproducing the infection in small animals and in recovering and identifying these parasites from these animals. This was the first description of the causative agent of American trypanosomiasis, *Trypanosoma cruzi*, named after his friend and mentor, Oswaldo Cruz. By reading the historical records, I realized that Chagas' work is one of the most complete and detailed descriptions of a new pathogen together with the consequent pathology in the history of science. In the year 1909, Chagas published the systematic description of the parasite and its development in the vector, the clinical development of *T. cruzi* in the intermediate and definitive hosts, his attempts to culture the parasite in the laboratory and the course of infection in experimental animals [49,50]. Two years later, he published a complete description of the human pathology known as Chagas' disease [51].

By mid-1960s, Hoare and Wallace used peculiarities in morphology and life cycle to redefine the taxonomic system of Trypanosomatidae [52]. Currently, the number of named species of insect trypanosomatids is close to 400 [38], although this number is certainly underestimated. A study from 2001 analyzed 2'500 insect hosts and found a few hundred of new presumptive species of trypanosomatids, supporting that the group is immensely diverse [53]. Nonetheless, from the 12 potential subfamilies of trypanosomes proposed in Maslov *et al*. [38], all of them are infectious to insects (with few exceptions: see Chapter 1.7) but only two include agents of human diseases, i.e. *Trypanosoma* clade and Leishmaniinae subfamily [38], showing that evolution of trypanosomatids is closely connected to their insect hosts. However, an impressive example of the plasticity of trypanosomes is the existence of monoxenic species that lost their capacity to infect the invertebrate host, becoming infectious only to mammals; these are *T. equiperdum*, causative agent of a horse disease called dourine, and *T. evansi*, a pathogenic parasite able to infect many different mammals causing a disease most frequently known as surra.

1.7 Dyskinetoplastic (DK) trypanosomes

T. evansi and *T. equiperdum* have adapted to live without parts or the whole mitochondrial DNA (kinetoplast, kDNA); thus, they are known as dyskinetoplastic (DK) trypanosomes. Since there are no vectors spreading the infection of these two agents, they are mechanically transmitted, and thus, are not restricted to the areas where insect vectors are present [54]. The transmission of *T.*

equiperdum is mainly by sexual route and *T. evansi* by biting flies [55]. The natural emergence of these two species stimulates discussions in the field of evolution of trypanosomes, e.g. there are diverging opinions whether or not *T. equiperdum* and *T. evansi* should be considered sub-species of *T. brucei* [54,56]. Nevertheless, the study of these atypical species of trypanosomes brought recent astonishing results that are contributing to the understanding of not only evolutionary concepts but also to the general biology of trypanosomes, mitochondrial function and drug development against trypanosomiases. The mainstay for this breakthrough was caused by the discussion about the function of kDNA in bloodstream form parasites [57] and how *T. equiperdum* and *T. evansi* have naturally lost their kDNA [58]. Since this topic is one of the most inspiring stories in the field of trypanosomes, I would like to briefly cover it.

kDNA of *T. brucei* contains 20-50 copies of maxicircles (~23 kb, Figure 7), which encode a typical set of rRNA and protein-coding genes, including subunits of the respiratory chain [1,58]. Expression of most of genes present in maxicircles requires a unique form of post-transcriptional editing before translation in functional proteins [57]. Guide RNAs are involved in regulation of this individual editing and are encoded by minicircles of kDNA, which are structures of ~1-kb with tremendous diversity in *T. brucei* (Figure 7).

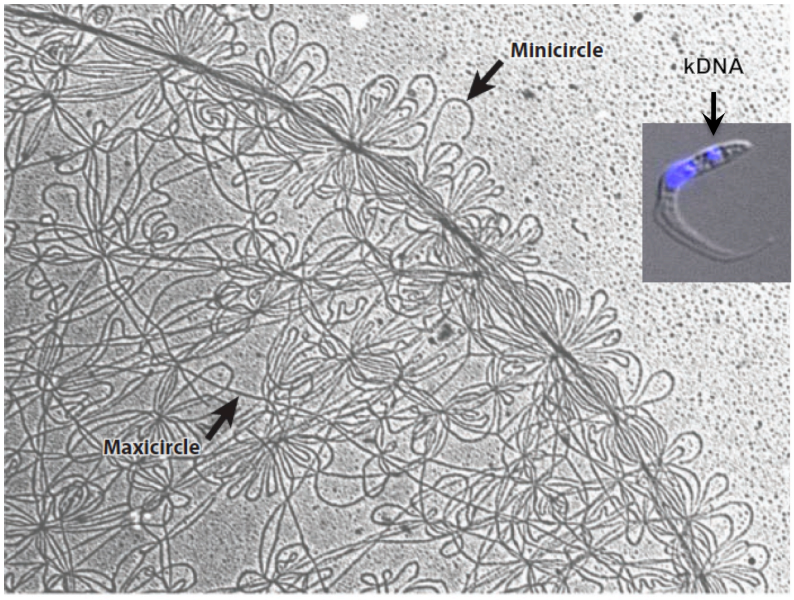

Figure 7. Electron microscopy picture of an isolated kDNA network from *Chritidia fasciculate*. The long strands of DNA are maxicircles and the small loops are minicircles. Reproduced from [1]. *Inset*, immunofluorescence picture (self-taken) of procyclic form of *T. brucei* stained with DAPI illustrating the position of kDNA.

T. equiperdum strains normally still contain their maxicircles but have lost their minicircle diversity, while *T. evansi* strains don't have maxicircles and can either present minicircle homogeneity or are akinetoplastic (lost completely all kDNA) [54,58,59]. Consequently, both species are unable to express functional mitochondrial genes and differentiate into insect-stage forms.

In contrast to *T. equiperdum* and *T. evansi*, the kDNA is essential for bloodstream forms of *T. brucei*. The kDNA encodes for the subunit A6 of the F_o component of F_1F_o-ATP synthase in the mitochondrion [58]. In bloodstream forms, the F_1F_o-ATP synthase is working in the reverse direction, pumping protons from mitochondrial matrix to the intermembrane space generating the mitochondrial membrane potential ($\Delta\Psi_m$) [60,61,62]. Thus, although bloodstream form parasites do not generate ATP via oxidative phosphorylation, they are still depending on F_1F_o-ATP synthase to generate the $\Delta\Psi_m$ [62,63]. This raises an intriguing question: how can dyskinetoplastic parasites be viable? The answer came years after the clever hypothesis discussed in [58], which was elaborated based on two observations: I) drug-induced dyskinetoplastic bloodstream form trypanosomes are viable and, thus, still possess the $\Delta\Psi_m$ necessary for mitochondrial function in *T. brucei*; and II) there were examples in yeast and mammalian cells lacking the mitochondrial genome, and consequently subunits of the ATP synthase, which were still able to generate a $\Delta\Psi_m$ because of rare compensatory mutations in nuclear-DNA-encoded subunits of the ATP synthase. Hence, Schnaufer *et al.* proposed compensatory mutations might also be present in *T. brucei* to allow a functional ATP synthase in absence of kDNA [58]. Recently, and several years after the initial hypothesis, the very exciting finding of Dean *et al.* elucidated the compensatory mechanism for the loss of kDNA [59]. They demonstrated that a point mutation in the nuclear-DNA-encoded gene of the γ subunit of the F_1 component of F_1F_o-ATP synthase can compensate for complete loss of kDNA in *T. brucei* [59]. Furthermore, they established a potentially interesting strain of *T. brucei* that tolerates loss of kDNA and can be used for the study of drug action, and essential mechanisms of replication and maintenance of kDNA. In Chapter 3.1 of this thesis, I have used this cell line to investigate the mode of action of choline analogs.

1.8 Choline transport systems

Trypanosomes need to build membranes to divide and keep proliferating. For this reason, they need to scavenge lipid precursors in large amounts to synthesize membrane lipids. One of the most required lipid precursors in eukaryotic cells is choline, which is used as a head group of phosphatidylcholine (PC), an abundant membrane glycerophospholipid (covered in the next topic), and of sphingomyelin (SM) [64]. Therefore, as discussed later in this introduction, mechanisms behind the synthesis of choline-containing lipids can be considered potential drug targets. First, I

describe in more details the different systems for choline uptake in human cells, the reference used to initiate our studies on choline uptake in trypanosomes.

Choline is a quaternary amine (trimethyl-β-hydroxy-ethylammonium, Figure 8), which is an essential nutrient for eukaryotic cells. In humans, it plays a role not only in the synthesis of PC and SM but also as a methyl-group donor in methionine metabolism [65] as well as in synthesis of the neurotransmitter acetylcholine [66,67]. Laboratory animals fed with a choline-deficient diet develop spontaneous carcinoma of the liver and generally increase sensitivity to carcinogens [68]. It is the only type of nutritional deficiency known to have such deleterious effect [69].

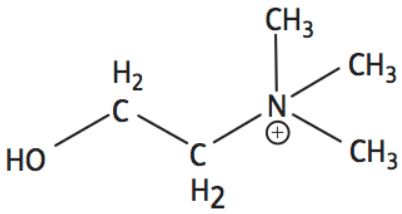

Figure 8. Chemical structure of choline. Taken from [70].

As a positively charged quaternary ammonium, choline cannot cross biological membranes. Characterization of choline uptake in human cells using radiolabeled choline and hemicholinium-3 (HC-3), the best characterized inhibitor of choline uptake [71], identified three different transport systems: I) low-affinity non-specific transport, II) high-affinity Na^+-dependent transport, and III) intermediate-affinity Na^+-independent transport [72]. All three systems are carrier-mediated, i.e. they follow Michaelis-Menten kinetics.

The low-affinity non-specific transport system is represented by the polyspecific organic cation transporter (OCT) family, comprising of five members that are present in different organs of the human body (Table 1). OCT proteins are not specific for choline and are not sensitive to HC-3. The physiological role of OCT proteins in choline transport has not been firmly established. OCT1 was shown to transport choline when transiently expressed in HEK cells [73]. Additionally, expression of OCT1 and OCT2 in *Xenopus* oocytes mediated choline uptake with low affinity, i.e. with K_m values of 346 µM for rat OCT1, 441 µM for rat OCT2, and 102 µM for human OCT2 [74].

High-affinity choline transport (CHT)	•K_m ≈ 0.5 to 3 μM •Na^+-dependent •Hemicholinium-3 sensitive (K_i ≈ 1-3 μM) •Energy-dependent
Intermediate-affinity choline transport (CTL)	•Choline specific and Na^+-independent •K_m ≈ 20-200 μM •Hemicholinium-3 sensitive (K_i ≈ 20-200 μM) •Energy-dependent
Low-affinity choline transport (OCT)	•Nonspecific •K_m > 300 μM •Not sensitive to hemicholinium-3

Table 1. Different systems for choline transport in humans. Adapted from [72].

The high-affinity choline transport system has a K_m value for choline at 0.5-3 μM and is sensitive to low concentrations of HC-3, with K_i values at ~1-3 μM [75] (Table 1). In addition, choline transport is energy- and Na^+-dependent. High-affinity choline transport is linked to acetylcholine biosynthesis in neurons [76,77,78]. The best-known representative of this transport system in humans is the protein named CHT1, which is expressed in cholinergic neurons [79,80].

The third transport system, intermediate-affinity choline transport, has K_m values of 20-200 μM and is less effectively inhibited by HC-3, showing K_i values of 20-200 μM. It is Na^+-independent and demonstrates specificity to choline [72] (Table 1). It is represented by the choline transporter-like (CTL) family, which includes five members (CTL1-5), with CTL1 being the most thoroughly studied [81]. CTL1 was initially shown to restore choline transport in choline transport-deficient yeast [82]. Furthermore, expression of *Torpedo* CTL1 in *Xenopus* oocytes increased Na^+-independent high-affinity choline uptake [82]. CTL proteins contain 10 highly conserved transmembrane domains (TMDs), with their N- and C-termini likely localized intracellularly (Figure 9). They are expressed in multiple human tissues and mediate uptake of choline for PC synthesis [72].

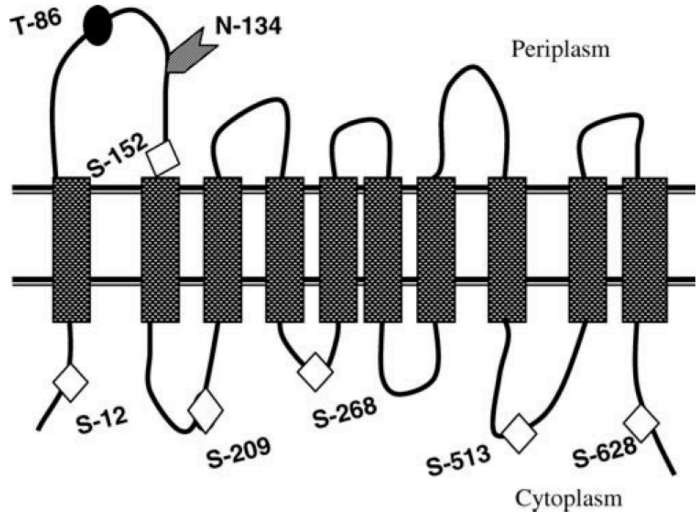

Figure 9. Representation of mouse CTL1 with 10 transmembrane domains with a large and variable loop between transmembrane domains 1 and 2, which is potentially glycosylated. Figure copied from [72].

To date, no choline transporter has been identified in any protozoa. However, it has been demonstrated that acquisition of choline is carrier-mediated in *Plasmodium* [83] and *Leishmania* [84]. Moreover, characterization of choline uptake into free intraerythrocytic stages of *Plasmodium falciparum* indicated an intermediate-affinity transport system (K_m of ~25 μM) that does not depend on sodium ions [83]. In *Leishmania major*, uptake of choline was shown to be specific for choline with a low micromolar K_m (=2.5 μM), and also Na^+-independent [84]. Hence, some features of choline transport in *Plasmodium* and *Leishmania* suggest the presence of a system related to the human ubiquitous CTL proteins, i.e. a Na^+-independent, intermediate-affinity choline uptake system [72]. Our work showed - for the first time - that a related system is also present in *T. brucei* [85]. Therefore, we reasoned that choline carrier in trypanosomes could be related to the human CTL proteins and, by *in silico* search, we found several choline transporter candidates in *T. brucei* genome. We empirically investigated the effect of down-regulation of these candidates by RNAi in different conditions and expressed some of them in heterologous systems but, unfortunately, the results were inconclusive [85] (manuscript also found in Chapter 2.1).

1.9 Phosphatidylcholine (PC) biosynthesis in trypanosomes

Membrane lipids are divided in three major groups: I) phospholipids comprise of glycerophospholipids, which are related to phosphatidic acid (Figure 10), and sphingophospholipids; II) sterols comprise of polycyclic compounds like cholesterol, the most abundant sterol in animals, the function of which is associated with fluidity of membranes; III) glycolipids, characterized by the

presence of single or multiple sugar moieties, are not ubiquitous being present in more representative amounts in brain tissue of animals and in chloroplast of plant cells [64].

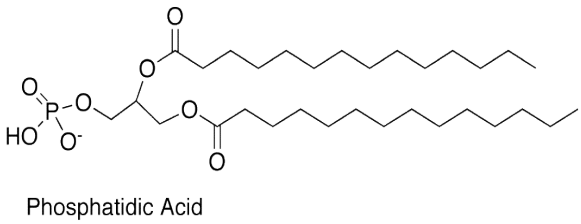

Figure 10. Chemical structure of phosphatidic acid, the precursor of glycerophospholipids.

The phospholipid composition of *T. brucei* bloodstream and procyclic forms is comparable to that other eukaryotic cell. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) represent the most abundant glycerophospholipids, comprising 45–60% and 10–20%, respectively, of total phospholipids (Figure 11). Phosphatidylinositol (PI), phosphatidylserine (PS) and cardiolipin (CL) represent minor glycerophospholipid classes and account for 6–12%, <4% and <3%, respectively [86]. Taken that into account, metabolism of PC, the most abundant phospholipid, is a critical process for any fast growing eukaryotic cell, including trypanosomes.

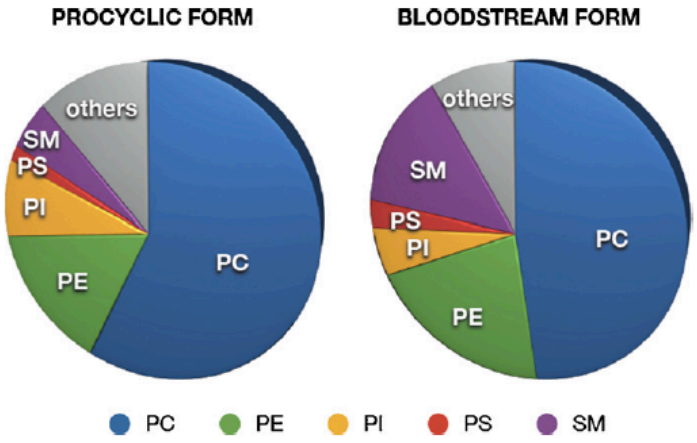

Figure 11. Relative distribution of phospholipid classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM), in *T. brucei* procyclic and bloodstream forms. Figure taken from [86].

PC is synthesized by two different routes in trypanosomes: I) it can be formed using lyso-phosphatidylcholine (lyso-PC) as a precursor, which is taken up and acylated to PC [87]; or II) it can be formed via the CDP-choline branch of the Kennedy pathway [88] using choline and diacylglycerol (DAG) as precursors [89].

By definition, lyso-phospholipids (lyso-PLs) are characterized by a single acyl chain and a polar head group, which in the case of lyso-PC is choline. It has been demonstrated that trypanosomes metabolize lyso-PLs more rapidly than their corresponding phospholipids [90]. Possibly because of the simpler chemical structure of lyso-PC, which renders these lipids more hydrophilic and versatile compared to their corresponding phospholipids. Because the conversion of lyso-PC to PC is rapid (only one CoA-dependent acylation is necessary, see Figure 12) and high concentrations of lyso-PC is present in human blood (about 200-300 μM [91,92], i.e. about 10x higher than free choline [93]), lyso-PC is likely the main route for synthesis of PC in bloodstream form parasites [89]. In contrast, however, marginal uptake of choline has been shown in bloodstream form trypanosomes isolated from rats [89].

Synthesis of PC from free choline occurs via the Kennedy pathway, named after Eugene P. Kennedy, who, together with Samuel B. Weiss, first described this pathway as a crucial route for biosynthesis of glycerophospholipids in all eukaryotic cells [88]. The Kennedy pathway (Figure 12) is divided into two branches, the CDP-choline branch that leads to the synthesis of PC and the CDP-ethanolamine branch, leading to PE synthesis. In the CDP-choline branch, free choline is phosphorylated by choline kinase and subsequently activated into CDP-choline by choline-phosphate citidyltransferase. Finally, activated choline is transferred to diacylglycerol by choline/ethanolamine phosphotransferase (CEPT), forming PC (Figure 12). Note that CEPT has been characterized and shows dual specificity for CDP-choline and CDP-ethanolamine, and was shown to be essential for growth of *T. brucei* in culture [94]. The CDP-ethanolamine pathway involves the activity of ethanolamine kinase and ethanolamine-phosphate cytidylyltransferase to phosphorylate ethanolamine and activate into CDP-ethanolamine, respectively. Ethanolamine phosphotransferase (EPT) catalyzes the final step leading to the formation of PE. Interestingly, PE produced by EPT comprises of alk-1-enyl-acyl-type species whereas the synthesis of PE by CEPT produces diacyl-type species of PE (Figure 12), suggesting that the two enzymes may be located in different compartments in *T. brucei* [94] and have access to different precursors.

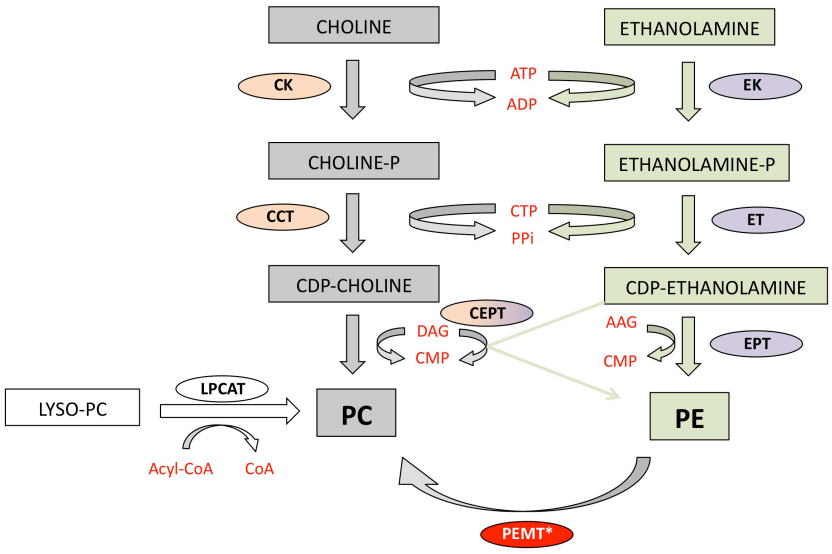

Figure 12. Synthesis of PC and PE by *T. brucei* via Kennedy pathway. Substrates from CDP-choline branch is represented in gray and involved enzymes in orange. CDP-ethanolamine branch is in green and purple. CEPT shows dual specificity (orange and purple) with higher affinity to CDP-choline. *Not present in *T. brucei*. Abbreviations: CK, choline kinase; CCT, cholinephosphate cytidyltransferase; CEPT, choline/ethanolamine phosphotransferase; EK, ethanolamine kinase; ET, ethanolamine cytidyltransferase; ethanolamine phosphotransferase; PEMT, phosphatidylethanolamine N-methyltransferase; LPCAT, lysophosphatidylcholine acyltransferase; AAG, alk-1-enyl-acylglycerol; DAG, diacylglycerol.

There is an alternative route to form PC that depends on the activity of phosphatidylethanolamine *N*-methyltrasferases, which catalyze the methylation of PE to PC [95] (PEMT, Figure 12). A homolog of this enzyme is present in *Leishmania* but so far it has not been characterized experimentally [96]. In contrast, no homolog has been found in *the T. brucei* genome. In addition, labeling of *T. brucei* lipids using radiolabeled ethanolamine or serine did not result in the formation of radioactive PC [94], indicating that trypanosomes do not have this pathway. Hence, *T. brucei* likely relies only on the Kennedy pathway for *de novo* PC synthesis.

1.10 Choline analogs: development and current knowledge

In trypanosomes, as well as in Plasmodium, there is a huge demand for lipid precursors to permit parasite growth within the human host. As in trypanosomes, PC represents the most abundant lipid in plasmodial membranes. Thus, pathways involved in PC synthesis are potential drug targets against sleeping sickness and malaria. In Plasmodium-infected erythrocytes, uptake of choline is substantially increased compared to erythrocytes from healthy controls [97]. Inhibition of this process was shown to be deleterious for the parasite [98]. Thus, during nearly two decades, a large

number of choline analogs have been screened for their potential to interfere with choline metabolism and PC synthesis in Plasmodium (reviewed in [98]). The researchers involved in the design and synthesis of these compounds chose a stepwise strategy of structure-activity-relationships (SARs) to optimize the their potency and, in a later stage of the study, to promote their pharmacodynamics in mammals [99]. Along the study, a group of compounds classified as bis-quaternary ammonium salts, with the leading compound named G25 (Figure 13), was found to have extremely high efficiency against Plasmodium, both *in vitro* [99] and *in vivo* [100]. However, toxicity and bioavailability of bis-quaternary ammonium salts in mammals were not satisfactory. Therefore, the quaternary ammonium ring was replaced by a thiazolium ring, present in Vitamin B1, to reduce its toxicity and increase oral absorption, leading to the second generation of compounds called bis-thiazolium salts [101] (Figure 13). In a new screening approach, two of these compounds, named T3 and T4, turned out to be superior to currently available anti-malarials in animal studies [102]. At present, T3 is in phase II of clinical trials since 2008 to treat severe malaria [103]. Subsequently, a third generation of compounds, including a group of 2-amino pyridinium salts and bis-alkylamidines, with the leading compounds being MS1, M38 and M53, were designed to minimize side effects, mainly in the CNS [104].

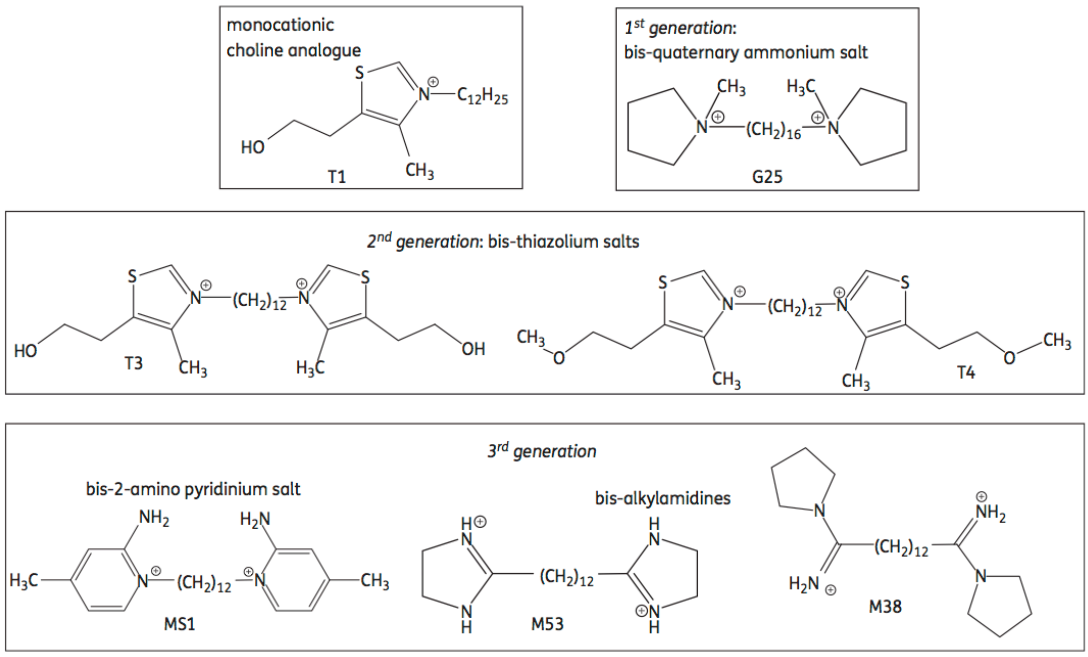

Figure 13. Chemical structures of anti-malarial choline analogs. Adapted figured reproduced from [70].

The most accepted view of the mode of action of choline analogs in Plasmodium is the specific inhibition of PC synthesis, which at least in part, may be due to inhibition of choline uptake

into the parasite [100,105,106,107]. Additionally, there is evidence that bis-thiazolium salts, T16 [83] and T3 [103], are transported by the Plasmodium choline carrier. More recently, *in vitro* studies showed that selected compounds belonging to the different groups of choline analogs also display high toxicity to *T. brucei* bloodstream forms [70]. The tests were also carried out using strains resistant against diamidines and arsenicals and showed little or no significant increase in resistance to choline analogs, indicating the absence of cross-resistance [70]. Phospholipid analyses suggested that choline analogs didn't affect the lipid content in *T. brucei* [70], contrasting with the reported mode of action in Plasmodium. Very recently, our own work showed that certain choline analogs are potent inhibitors of choline uptake in *T. brucei* [85], however, inhibition kinetics suggested that choline uptake was not the main target of these compounds since they displayed high toxicity even when used in much lower concentrations than the IC_{50} values for inhibition of choline uptake [85] (manuscript found in Chapter 2.1). Alternatively, choline analogs were proposed to affect mitochondria by decreasing the $\Delta\Psi_m$ in *T. brucei,* leading to parasite death in culture [70].

1.11 The mitochondrion of T. brucei

Stumpy form trypanosomes encounter a very different environment when they are taken up from the mammalian host into the tsetse fly midgut. To adapt to the change in nutrient availability, the rudimentary mitochondrion of bloodstream form trypanosomes undergoes dramatic alterations during parasite differentiation to procyclic forms [108,109]. The most striking change occurs in mitochondrial energy production. Long slender bloodstream forms have a very simple energy metabolism, relying mostly on glycolysis for ATP production [110,111]. In special organelles called glycosomes, glucose is degraded to 3-phosphoglycerate, which is then further metabolized in the cytosol to pyruvate, the excreted metabolic end product. Pyruvate formation is catalyzed by pyruvate kinase (number 12 in Figure 14), which uses phospho*enol*pyruvate (PEP) and ADP as substrates producing pyruvate and ATP [112], thus, no net ATP synthesis occurs in the glycosome. The redox balance within the glycosome is maintained by a glycerol-3-phosphate shuttle, together with glycerol-3-phosphate oxidase (number 38 in Figure 14) present in the outer leaflet of inner mitochondrial membrane [113]. Bloodstream forms parasites can sustain this pathway for energy production because they live in a nourishing environment in the mammalian blood that contains high concentrations of glucose.

However, it should be noted that even though energy metabolism in bloodstream form parasites does not involve oxidative phosphorylation, mitochondrial function and biogenesis are essential at this stage of the parasite life cycle [60]. In particular, the mitochondrion plays an essential role by providing reducing agents to maintain the glycosomal redox balance [113] (see

above), maintaining mitochondrial translation [114], regulating mtDNA expression [58], and for mitochondrial fatty acid synthesis [115]. The electrochemical proton gradient ($\Delta\Psi_m$) is essential to energize translocation of proteins and transport co-factors across the inner mitochondrial membrane to maintain mitochondrial function [114,116]. The mitochondrion of *T. brucei* bloodstream forms lacks complexes III and IV of the respiratory chain, thus the $\Delta\Psi_m$ is generated by the activity of the F_1F_o-ATP synthase, which is working in the reverse direction, i.e. pumping protons from mitochondrial matrix to the intermembrane space [60,61,62].

In contrast, in procyclic forms, the end product of glycolysis, pyruvate, is not excreted but is further metabolized in the mitochondrion and degraded mainly to acetate. Acetate production is catalyzed by acetate:succinate CoA-transferase (ASCT, number 24 in Figure 14) and involves a succinate/succinyl-CoA cycle that generates ATP by substrate level phosphorylation [117]. In the tsetse midgut, glucose supply is very limited, except immediately after a blood meal when the glucose concentration increases and is quickly consumed by the insect (~15 minutes, [118]). In contrast, proline is an abundant carbon source in the tsetse midgut [119,120], what led some authors to speculate more than three decades ago that this amino acid is a key source of energy for the insect forms of *T. brucei* [121,122]. But only a compelling study by Lamour *et al*. less than a decade ago has systematically investigated the metabolism of proline, elucidating its role in energy metabolism of procyclic forms [123]. The same study demonstrated that glucose, if available, is the preferred carbon source of procyclic forms, however in glucose-depleted conditions uptake of proline for energy production is 6-fold increased [123].

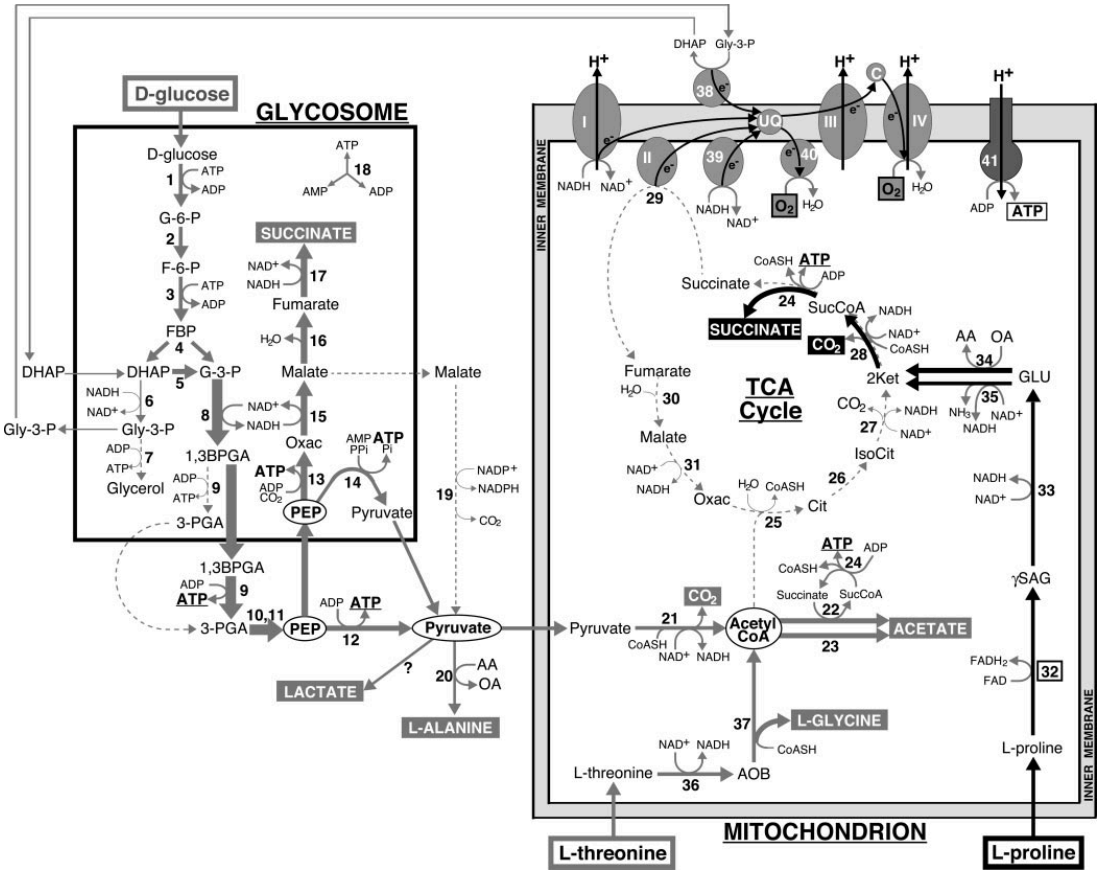

Figure 14. Schematic representation of carbon sources in *T. brucei*. Figure and legend were reproduced from [123].

Abbreviations: *AA*, amino acid; *AOB*, amino oxobutyrate; *1,3BPGA*,1,3-bisphosphoglycerate; *C*, cytochrome *c*; *Cit*, citrate; *CoASH*, coenzyme A; *DHAP*, dihydroxyacetone phosphate; *F-6-P*, fructose 6-phosphate; *FBP*,fructose 1,6-bisphosphate; *G-3-P*, glyceraldehyde 3-phosphate; *G-6-P*, glucose 6-phosphate; *GLU*, glutamate; *Gly-3-P*, glycerol 3-phosphate; *IsoCit*,isocitrate; *2Ket*, 2-ketoglutarate; *OA*, 2-oxoacid; *Oxac*, oxaloacetate; 3-*PGA*, 3-phosphoglycerate; *Pi*, inorganic phosphate; *PPi*, inorganic pyrophosphate; *_SAG*, glutamate _-semialdehyde; *SucCoA*, succinyl-CoA; *UQ*, ubiquinone pool. Enzymes are as follows: *1*, hexokinase: *2*, glucose-6-phosphate isomerase; *3*, phosphofructokinase; *4*, aldolase; *5*, triose-phosphate isomerase; *6*, glycerol-3-phosphate dehydrogenase; *7*, glycerol kinase;*8*, glyceraldehyde-3-phosphate dehydrogenase; *9*, phosphoglycerate kinase; *10*, phosphoglycerate mutase; *11*, enolase; *12*, pyruvate kinase; *13*,phospho*enol*pyruvate carboxykinase; 14, pyruvate phosphate dikinase; *15*, glycosomal malate dehydrogenase; *16*, glycosomal fumarase; *17*,NADH-dependent fumarate reductase; *18*, glycosomal adenylate kinase; *19*, malic enzyme; *20*, alanine aminotransferase; *21*, pyruvate dehydrogenasecomplex; *22*, acetate:succinate CoA-transferase; *23*, unknown enzyme, possibly acetyl-CoA synthetase; *24*, succinyl-CoA synthetase; *25*, citrate synthase; *26*, aconitase; *27*, isocitrate dehydrogenase; *28*, 2-ketoglutarate dehydrogenase complex; *29*, succinate dehydrogenase (complexII of the respiratory chain); *30*, mitochondrial fumarase; *31*, mitochondrial malate dehydrogenase; *32*, proline dehydrogenase; *33*, pyrroline-5carboxylate dehydrogenase; *34*, glutamate aminotransferase; *35*, glutamate dehydrogenase; *36*, L-threonine dehydrogenase; *37*, acetyl-CoA:glycine*C*-acetyltransferase; *38*, glycerol-3-phosphate oxidase; *39*, rotenone-insensitive NADH dehydrogenase; *40*, alternative oxidase; *41*, F0/F1-ATPsynthase; *I–IV*, complexes of the respiratory chain.

When cultivated in standard medium (i.e. in the presence of 6 mM glucose), substrate level phosphorylation is the main route for ATP production by mitochondria of procyclic forms and, in consequence, respiratory chain and F_1F_o-ATP synthase activities are down-regulated [124]. Accordingly, oxidative phosphorylation is not strictly essential in procyclic forms, as was demonstrated by inhibition of the ATP synthase by oligomycin, resulting in a decrease in cell growth, but not in death of the parasite [123]. In contrast, when experiments were conducted in glucose-depleted conditions, sensitivity to oligomycin increased 2'000-5'000 times, suggesting that when procyclic forms switch to proline metabolism oxidative phosphorylation becomes essential [123,125].

1.12 Mitochondrial carrier family (MCF)

The inner mitochondrial membrane is impermeable to metabolites indicating the existence of a number of carriers that contribute to the constant traffic between different cell compartments, i.e. mitochondrial matrix and cytosol [126]. A group of carriers involved in this traffic belongs to the mitochondrial carrier family (MCF), which are structurally related mitochondrial proteins responsible for the transport of mono-, di-, and tricarboxylates, inorganic phosphates and nucleotides, co-factors such as FAD and NAD^+, or cations or zwitterions such as ornithine, carnitine, spermidine and glutamine [127]. The primary structure of the carriers consists of three repetitions in tandem of about 100 amino acids in length with two alpha helices connected by a hydrophilic loop and a signature sequence [128,129]. Both the carboxy- and amino-terminal ends of the MCF proteins are exposed to the intermembrane space [128]. The structure of the bovine ATP/ADP carrier has been resolved with 2.2 Å resolution [130] and brought new insights in the understanding of the structure of MCF members: i) it exhibits a three-fold pseudo-symmetry in line with the sequence repeat described above [129]; ii) the structure has been highly used as a template for building homology models for various carriers [127].

The genome of *Saccharomyces cerevisiae* was predicted to encode 34 MCF proteins [131], whereas the *Arabidopsis thaliana* [132,133] and human genome [134] were predicted to encode 58 and 67 MCF proteins, respectively. A search of the *T. brucei*, *T. cruzi* and *L. major* genomes predicted 24 putative MCF proteins with significant homology to mitochondrial carriers from other eukaryotes [135]. Comparisons to previously described and functionally characterized MCF proteins from different eukaryotes allowed to assign putative function to most of the 24 MCF members in *T. brucei*. However, functional studies have been done for only two of the *T. brucei* MCF proteins [136]. Very recently, we have used a previously established RNAi library [137] to screen for genes in the *T. brucei* genome involved in the mode of action of choline analogs known as anti-malarials (described in Chapter 1.10). We identified a member of MCF, TbMCP14, which brought new insights about the

anti-kinetoplast effects of these choline analogs as well as the function of this novel mitochondrial carrier.

2. Results

2.1 Characterization of choline uptake in Trypanosoma brucei procyclic and bloodstream forms

(Published manuscript)

Author contributions:

R. Schmidt constructed some of the cell lines initially used in the study;

P. Mäser made the bioinformatic analyses that identified the CTL homologs in *Trypanosoma* genome;

D. Rentsch contributed with advices on yeast experiments;

E. Sigel contributed with advices on *Xenopus* oocyte experiments;

H. Vial provided the choline analogs;

All experimental data and figures on this paper were generated by myself.

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Characterization of choline uptake in *Trypanosoma brucei* procyclic and bloodstream forms

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ABSTRACT

Choline is an essential nutrient for eukaryotic cells, where it is used as precursor for the synthesis of choline-containing phospholipids, such as phosphatidylcholine (PC). According to published data, *Trypanosoma brucei* parasites are unable to take up choline from the environment but instead use *lyso*-phosphatidylcholine as precursor for choline lipid synthesis. We now show that *T. brucei* procyclic forms in culture readily incorporate [^{3}H]-labeled choline into PC, indicating that trypanosomes express a transporter for choline at the plasma membrane. Characterization of the transport system in *T. brucei* procyclic and bloodstream forms shows that uptake of choline is independent of sodium and potassium ions and occurs with a K_m in the low micromolar range. In addition, we demonstrate that choline uptake can be blocked by the known choline transport inhibitor, hemicholinium-3, and by synthetic choline analogs that have been established as anti-malarials. Together, our results show that *T. brucei* parasites express an uptake system for choline and that exogenous choline is used for PC synthesis.

1. Introduction

By definition, endoparasites scavenge nutrients from their hosts. Among the essential nutrients for kinetoplastids and apicomplexans are precursors like choline, ethanolamine and *myo*-inositol for the synthesis of phospholipids, the major structural components of membranes in protozoan parasites [1].

In *Trypanosoma brucei*, the causative agent of human African sleeping sickness and the related animal disease, Nagana, phosphatidylcholine (PC) represents the most abundant phospholipid class, representing 45–60% of total phospholipid [2]. The formation of PC can be initiated by uptake of *lyso*-phosphatidylcholine (*lyso*-PC) from the environment, followed by CoA-dependent acylation to PC [3]. This pathway has been shown to contribute most of the PC in *T. brucei* bloodstream forms [4]. Alternatively, PC can be synthesized de novo via the CDP-choline branch of the Kennedy pathway, using choline as precursor [4]. The enzymes of the CDP-choline pathway have been identified and experimentally verified in *T. brucei* [5,6]. In addition, RNAi studies revealed that the pathway is essential in both *T. brucei* bloodstream [1] and procyclic [6] forms. In contrast, little and conflicting data are available on the acquisition of the precursor molecule, choline, for de novo PC synthesis via the CDP-choline pathway. Marginal uptake of choline has been reported in *T. brucei* bloodstream forms isolated from rats [4], however, this finding was not confirmed in more recent studies [7,8].

Choline uptake in mammals is mediated via several distinct transport systems. Based on ion dependence and susceptibility to the specific inhibitor, hemicholinium-3 (HC-3) [9], choline transport can occur via (i) low-affinity facilitated diffusion, mediated by polyspecific organic cation transporters, (ii) intermediate-affinity, sodium-independent transport, using choline transporter-like (CTL) proteins, and (iii) high-affinity, sodium-dependent transport, catalyzed by high-affinity choline transporters [10]. In humans, organic cation transporters and CTL proteins are ubiquitously distributed. In contrast, high-affinity choline transporters primarily occur in cholinergic neurons, where they mediate uptake of choline for acetylcholine synthesis [11,12].

Choline transport systems have also been identified and characterized in *Plasmodium* and *Leishmania*. Uptake of choline in these

Abbreviations: PC, phosphatidylcholine; lyso-PC, lyso-phosphatidylcholine; HC-3, hemicholinium-3; CTL, choline transporter-like; TLC, thin layer chromatography.

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parasites is sodium-independent, can be inhibited by HC-3, and occurs with affinities in the low micromolar range (2.5–25 μM), suggesting that it is mediated by intermediate-affinity CTL proteins [13,14]. Interestingly, a group of synthetic choline analogs was shown to effectively inhibit choline uptake in both groups of parasites [7,14]. It has been suggested that in *Plasmodium* the drugs may act via interference with phospholipid biosynthesis [14–18]. Some of these choline analogs have been used as anti-malarials [15].

In the present study, we show for the first time that choline is readily taken up by *T. brucei* bloodstream and procyclic forms in culture. We characterize the transport system, which is independent of Na^+ and K^+ ions, and show that it is inhibited by HC-3 and synthetic choline analogs. Our analyses also demonstrate that exogenous choline is used by trypanosomes for PC synthesis.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade and purchased from Merck (Darmstadt, Germany), Sigma–Aldrich (Buchs, Switzerland) or ICN Biomedicals (Tägerig, Switzerland). Antibiotics and fetal bovine serum (FBS) were obtained from Invitrogen (Basel, Switzerland). DNA polymerases were purchased from Promega (Madison, USA). Primers and sequencing services were from Microsynth AG (Balgach, Switzerland). All restriction enzymes were purchased from Fermentas (Nunningen, Switzerland). methyl-[^{3}H]choline chloride (60 Ci/mmol; [^{3}H]choline), *myo*-[2-^{3}H(N)]inositol (15–20 Ci/mmol; [^{3}H]inositol) and [1-^{3}H]ethanolamine hydrochloride (40–60 Ci/mmol; [^{3}H]ethanolamine) were from American Radiolabeled Chemicals (St. Louis, USA), and [α-^{32}P]-dCTP (3000 Ci/mmol) from PerkinElmer Life Sciences (Schwerzenbach, Switzerland). Kodak MXB and BioMax MS films were from Kodak SA (Lausanne, Switzerland).

2.2. Trypanosomes and culture conditions

T. brucei strain Lister 427 procyclic forms were cultured at 27 °C in SDM-79 containing 5% (v/v) heat-inactivated FBS. *T. brucei* bloodstream and procyclic forms expressing T7 RNA polymerase and a tetracycline repressor [19] were cultured at 37 °C in HMI-9 containing 10% (v/v) heat-inactivated FBS and 27 °C in SDM-79 containing 15% (v/v) heat-inactivated FBS, respectively, in the presence of 25 μg/ml hygromycin, and 15 μg/ml G418. The derived clones containing different double-stranded RNA constructs against putative choline transporter genes were cultured in the presence of an additional 2 μg/ml puromycin or 1 μg/ml phleomycin. Generation of double-stranded RNA was induced by the addition of 1 μg/ml tetracycline.

2.3. Choline uptake in T. brucei

T. brucei procyclic and bloodstream forms were grown to a density of 1.0–1.5 x 10^7 and 1.0–1.5 x 10^6 cells/ml, respectively, washed once in 25 ml of assay buffer (130 mM NaCl, 10 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 5 mM NaH_2PO_4 and 5 mM glucose, pH 7.0) by centrifugation (1250 x *g*, 10 min, 24 °C) and resuspended in the same buffer, pre-warmed at 27 °C and 37 °C, respectively, at a density of 5 x 10^7 cells/ml. Parasites were then incubated for 3, 6 and 9 min in 96-well plates at room temperature (for procyclic forms) or 37 °C (for bloodstream forms) in the presence of 65 nM (0.8 μCi) [^{3}H]choline and 120 nM to 62.5 μM (final concentrations) non-labeled choline per well in a total volume of 200 μl. Uptake of [^{3}H]choline was terminated by rapidly transferring the samples to 96-well microplates containing 1.2 μM pore size GF/C glass fiber filters (UniFilter-96 GF/C, Perkin Elmer Life Sciences), immediately followed by filtration. After washing the pellets four times with 100 μl assay buffer, the samples were air-dried, resuspended in 40 μl scintillation fluid and counted in a 1450 MicroBeta Trilux top counter (Perkin Elmer Life Sciences). Uptake of [^{3}H]choline was linear over 9 min for all concentrations tested. The results from 4–5 independent experiments were used for calculation of K_m values, using Graphpad Prism 5 software and Lineweaver–Burk transformation. Choline uptake inhibition experiments were performed using 65 nM [^{3}H]choline and 1 μM non-labeled choline in the absence or presence of different concentrations of inhibitors. To study the ion dependence of choline uptake, trypanosomes were incubated in the absence of Na^+, K^+, or both ions, by replacing Na^+ for K^+, or vice versa (135 mM, final concentrations), or using 135 mM *N*-methylglucamine instead of Na^+ and K^+, respectively, in the assay buffer. No NaH_2PO_4 was used in these experiments.

2.4. Incorporation of [^{3}H]choline into T. brucei lipids

Metabolic labeling of trypanosomes with [^{3}H]choline and [^{3}H]ethanolamine (to control for cell viability and phospholipid synthesis) was done essentially as described before [20]. Briefly, [^{3}H]choline (1 μCi/ml) or a mixture of [^{3}H]choline and [^{3}H]ethanolamine (2 μCi/ml and 0.25 μCi/ml, respectively) was added to *T. brucei* procyclic forms at a density of 0.8–1.2 x 10^7 cells/ml and incubated in the absence or presence of G25 or MS1 (5 μM or 10 μM, respectively, final concentrations) for 2 h at 27 °C. Subsequently, parasites were spun down, washed with ice-cold buffer (10 mM Tris, 144 mM NaCl, pH 7.4) to remove unincorporated label, and phospholipids were extracted with 2 x 10 ml chloroform:methanol (2:1, by volume). Lipids were then separated by one-dimensional thin-layer chromatography (TLC) using Silica Gel 60 plates in a solvent system composed of chloroform:methanol:acetic acid:water (25:15:4:2, by volume). On each plate, appropriate phospholipid standards were run alongside the samples to be analyzed. Lipid spots were visualized by exposing the plates to iodine vapor. Radioactivity was detected by scanning the air-dried plates with a radioisotope detector (Berthold Technologies, Regensdorf, Switzerland) and quantified using the Rita Star® software provided by the manufacturer. Alternatively, plates were sprayed with EN^{3}HANCE (PerkinElmer Life Sciences) and exposed to MXB film at −70 °C.

2.5. Drug sensitivity assays

Susceptibility of *T. brucei* bloodstream forms to G25 was assessed by Alamar blue assays [21]. Briefly, serial dilutions of G25 starting at 10 μM (using a 10 mM stock solution) were prepared in HMI-9 medium containing 10% (v/v) FBS in 96-well plates (100 μl total volume). Parasites were added to a final density of 1 x 10^4 cells/ml. After incubation for 70 h at 37 °C, 10 μl of Alamar blue solution (12.5 mg resazurin/100 ml buffer) was added to all wells and incubation was continued for another 2 h at 37 °C. Fluorescence was measured using a spectromax GEMINI plate reader at 544 nm excitation, 590 nm emission and 570 nm cutoff.

2.6. In silico identification of genes encoding candidate choline transporters

All *T. brucei* proteins with two or more predicted [22] transmembrane domains were hierarchically clustered into similarity groups based on all pairwise global alignments [23]. For each cluster a hidden Markov model profile was made with ClustalW [24] and *hmmbuild* of the HMMer package [25]. The same procedure was carried out with the well-annotated proteomes of *Homo sapiens*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. Finally, the

pooled profiles were clustered based on HMM–HMM alignment carried out with HHsearch [26]. This allowed to tentatively annotate *T. brucei* multispanning transmembrane protein families of unknown function.

2.7. RNAi-mediated gene silencing

The nucleotide sequences of the genes encoding the putative CTL transporters, Tb927.10.6720, Tb927.10.6730, Tb927.10.6740, Tb927.10.6750 and Tb927.10.6760, show a high degree of identity and, thus, were targeted for down-regulation by RNAi using a single stem loop construct. In contrast, Tb927.10.530 shows no significant sequence homology to the other candidate CTL transporter genes and was down-regulated using a different construct. Cloning of the gene fragments into the tetracycline-inducible vector, pALC14 (a kind gift of André Schneider, University of Bern), was performed as described previously [27], using PCR products obtained with primers Tb6720-60-F (5′-GCCCAAGCTTGGATCCCGGCTCCATTTGCTTTGGG-3′) and Tb6720-60-R (5′-CGTCTAGACTCGAGTACACACGTGGAGGTGCTG-3′) (spanning nucleotides 924–1472 of Tb927.10.6730), resulting in plasmid pRS6720-60, and Tb530-F (5′-GCCCAAGCTTGGATCCACCTGATGAAGCCCGAGTG-3′) and Tb530-R (5′-CGTCTAGACTCGAGGCTGAGTGATGGTGGGAAG-3′) (spanning nucleotides 12–580 of Tb927.10.530) resulting in plasmid pJPM530. Plasmid extraction was performed using Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before transfection of *T. brucei* parasites, plasmid DNA was linearized with *Not*I, extracted with phenol and chloroform and precipitated with ethanol.

2.8. Stable transfections of trypanosomes

T. brucei 29-13 procyclic forms were harvested at mid-log phase, washed once in buffer (132 mM NaCl, 8 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM magnesium acetate, 0.09 mM calcium acetate, pH 7.0) and resuspended in the same buffer to a density of 8×10^7 cells/ml. Subsequently, 440 μl of parasite suspension were mixed with 15 μg of linearized plasmids pRS6720-60 or pJPM530 and transferred to a 0.2-cm pulse cuvette (Bio-Rad). Electroporation was conducted with a BTX Electroporation 600 system (Axon Lab, Baden, Switzerland) with one pulse (1.5 kV charging voltage, 2.5 kV resistance, 25 microfarads capacitance timing, and 186 resistance timing). Cells were immediately inoculated in 10 ml of procyclic or bloodstream form medium. Dilutions were plated into 24-well plates and after 24 h selected for antibiotic resistance by addition of 2 μg/ml puromycin. Clones were obtained by limiting dilution.

2.9. RNA isolation, quantitative PCR and Northern blot analysis

Total RNA was isolated using the SV Total RNA Isolation System (Promega), following the manufacturer's instructions. cDNA was synthesized from total RNA (0.1–0.5 μg) using SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR was performed using Applied Biosystems ViiA7 real-time PCR system. The reaction mixture consisted of 1x Fast Sybr Green master mix (Applied Biosystems, Switzerland) and 0.9 μM forward and reverse primers. For Northern blotting, total RNA (10–15 μg) was separated on formaldehyde–agarose gels (1% agarose, 2% formaldehyde in 20 mM Mops, containing 8 mM sodium acetate and 1 mM EDTA, pH 7.0) and transferred to Amersham Hybond-N^+ nylon membranes (GE Healthcare, Buckinghamshire, UK). The PCR products used to construct the RNAi stem loop vectors were used as templates to make the [^{32}P]-labeled probes by random priming, using Prime-a-Gene Labeling System (Promega). Hybridization was performed overnight at 60 °C in hybridization buffer containing 7% (w/v) SDS, 1 mM EDTA, 0.5 M Na_2HPO_4, 24 mM H_3PO_4, pH 7.2, and the membrane was analyzed by autoradiography using BioMax MS film and a TransScreen-HE intensifying screen. Ribosomal RNA was visualized on the same formaldehyde–agarose gel by ethidium bromide staining to control for equal loading.

2.10. Expression of candidate choline transporters in yeast and uptake assays

S. cerevisiae strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and the *hnm1* mutant strain Y04444 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YGL077c::kanMX4) were purchased from Euroscarf (Institute of Microbiology, University of Frankfurt). Transformation was performed as described [28] and transformants were selected on standard synthetic dextrose minimal medium supplemented with leucine (100 mg/l), histidine (20 mg/l) and methionine (20 mg/l). For uptake studies, yeast cells were grown to logarithmic phase, harvested at an OD_{600} of approximately 0.5, washed twice in water, and resuspended in buffer A (50 mM potassium phosphate, pH 5.0–7.5, containing 0.6 M sorbitol) to a final OD_{600} of 6. Prior to uptake measurements, cell suspensions were supplemented with 100 mM glucose and incubated for 5 min at 30 °C. To start the reaction, 130 μl of cell suspension were added to 130 μl of buffer A, containing 0.2 μCi [^{3}H]choline and 10 μM unlabeled choline. Samples were removed after 0.5, 1, 2, and 5 min, transferred to 4 ml of ice-cold buffer A, filtered on glass fiber filters and washed twice with 4 ml of ice-cold buffer A. Tritium uptake was determined by liquid scintillation counting.

3. Results

3.1. Choline uptake in T. brucei

In *T. brucei* bloodstream forms, uptake of choline for PC synthesis has been reported to be negligible, or absent [4,7]. We now found that incubation of *T. brucei* procyclic forms for 2 h in the presence of [^{3}H]choline resulted in the labeling of a major lipid class, as revealed by TLC analysis combined with fluorography and radioisotope scanning (Fig. 1). Co-migration with phospholipid standards

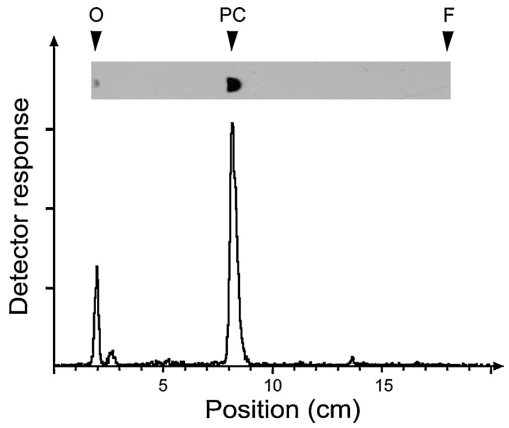

Fig. 1. Biosynthesis of [^{3}H]PC in *T. brucei*. Procyclic form trypanosomes (4×10^7 parasites) were incubated in the presence 1 μCi/ml [^{3}H]choline for 2 h in 5 ml culture medium. Phospholipids were extracted using organic solvents and separated by TLC. [^{3}H]-labeled lipids were analyzed by radioisotope scanning, and fluorography (inset). The arrows refer to the migration of a phosphatidylcholine (PC) standard run on the same TLC plate. The site of sample application (O) and the migration distance of the solvent front (F) are indicated.

applied on the same TLC plate and chromatography in several different solvent systems, including a two-dimensional separation system, identified the labeled lipid class as PC. Upon longer labeling and exposure times, a second minor [^{3}H]-labeled lipid class was detected (result not shown) and identified as sphingomyelin by co-migration with lipid standards. Similar observations were also made when *T. congolense* procyclic forms in culture were incubated in the presence of [^{3}H]choline (result not shown). Together, these findings suggest that [^{3}H]choline is taken up by *T. brucei* procyclic forms and used for the synthesis of choline-containing phospholipids.

To further characterize choline uptake in *T. brucei* procyclic forms, parasites were incubated in buffer containing [^{3}H]choline (65 nM, final concentration) in the presence of unlabeled choline (62.5 μM, final concentration) for up to 9 min. Determination of the amounts of radioactivity in parasites after rapid removal of the supernatant by filtration and repetitive washing showed a time-dependent linear increase in cell-bound [^{3}H]choline (Fig. 2A). To determine the kinetic parameters of choline uptake, parasites were incubated in the presence of trace amounts of [^{3}H]choline and increasing concentrations of unlabeled choline. For each choline concentration, samples were taken after 3, 6 and 9 min and showed linear increase in [^{3}H]choline uptake over time (results not shown; see Fig. 2A as an example). The data show that choline uptake follows Michaelis–Menten kinetics and is saturable (Fig. 2B). The combined results reveal that choline is taken up in *T. brucei* procyclic forms with a K_m of 2.0 ± 0.5 μM and V_{max} of 8.1 ± 2.1 pmol/min x 10^7 cells (mean values ± standard deviations of duplicate determinations from 5 separate experiments) (Fig. 2B and C). Choline uptake was temperature-dependent since no choline uptake was observed when parasites were incubated in the presence of [^{3}H]choline for 1 h on ice. Choline uptake also occurred linearly in *T. brucei* bloodstream forms in culture (Fig. 2D) and showed similar saturation kinetics (Fig. 2E). Under similar conditions as for procyclic trypanosomes, bloodstream form parasites took up choline with a K_m of 7.2 ± 4.8 μM and V_{max} of 5.7 ± 3.1 pmol/min x 10^7 cells (mean values ± standard deviations of duplicate determinations from 4 separate experiments) (Fig. 2E and F).

3.2. Characterization of choline uptake

Choline transport in *T. brucei* was further characterized by measuring uptake of [^{3}H]choline in procyclic forms in the presence of structurally related compounds. The results show that the choline transport inhibitor, HC-3 (50 μM, final concentration), reduced [^{3}H]choline uptake by 67%, while betaine, carnitine and ethanolamine had no effect (Fig. 3A). As positive control for competition, parasites were incubated in the presence of unlabeled choline (50 μM, final concentration), resulting in >90% inhibition of [^{3}H]choline uptake. In mammalian cells, sensitivity to HC-3 is often used to determine if choline uptake occurs via high, intermediate or low affinity transport [10]. Thus, we determined the affinity of the choline transport system to HC-3 by measuring uptake of choline (at 1 μM) in the presence of increasing concentrations of HC-3. Our results show that inhibition occurred with IC_{50} values of 27 ± 10 μM (Fig. 3B) and 61 ± 10 μM (not shown) in *T. brucei* procyclic and bloodstream forms, respectively.

Dependence of choline uptake on extracellular monovalent cations was studied by incubating *T. brucei* procyclic forms in buffer lacking Na^+, K^+, or both ions. The results show that the ion composition had little effect on choline uptake (Fig. 3C). In addition, the presence of high concentrations of the Na^+/K^+ pump inhibitor, ouabain (pre-incubated for 1 h at 2 mM, final concentration), had no effect on [^{3}H]choline uptake. In contrast, we found that addition of uncouplers of oxidative phosphorylation, 2,4-dinitrophenol (50–250 μM, final concentration) and CCCP (10 μM, final concentration), inhibited [^{3}H]choline uptake by 52.7–93.6% and 94.7%, respectively (values from three and two determinations, respectively). Together, these results indicate that choline transport in

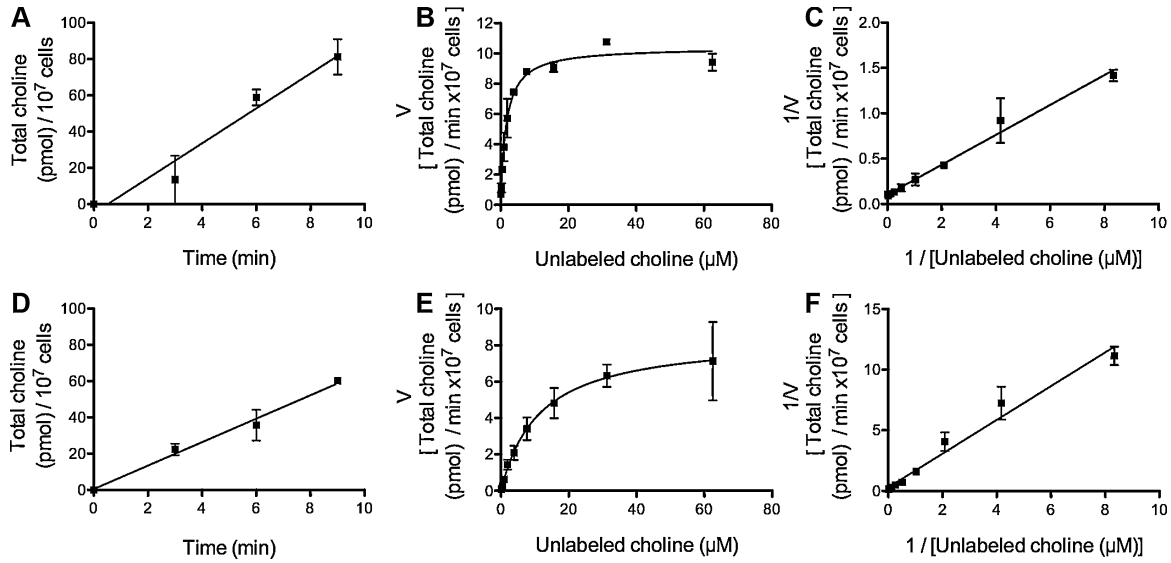

Fig. 2. K_m determination of choline uptake. Uptake of choline: *T. brucei* procyclic (panel A) and bloodstream (panel D) forms were incubated in assay buffer containing 65 nM [^{3}H]choline and 62.5 μM unlabeled choline for indicated times and the amounts of cell-bound radioactivity were determined by scintillation counting. K_m determination: *T. brucei* procyclic (B and C) and bloodstream (E and F) forms were incubated in the presence of increasing concentrations of choline (0.12–62.5 μM) for up to 9 min. At selected time points, uptake of radioactivity was determined and the results were analyzed according to Michaelis–Menten (B and E) or by Lineweaver–Burk transformation (panels C and F). Data points represent mean values ± standard deviations of duplicate determinations from two separate experiments done on the same day using parasites from the same culture flask.

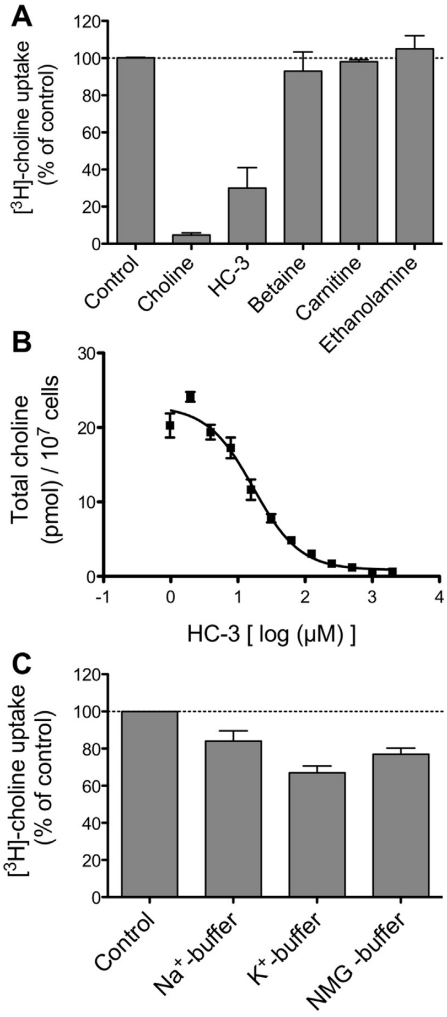

Fig. 3. Inhibition of choline uptake. *T. brucei* procyclic forms were incubated in buffer containing 65 nM [^{3}H]choline and 1 μM unlabeled choline for 10 min and the amounts of cell-bound radioactivity were determined by scintillation counting. Panel A: Inhibition of [^{3}H]choline uptake by the addition of unlabeled choline, hemicholinium-3 (HC-3), betaine, carnitine (each at 50 μM, final concentrations), and ethanolamine (100 μM, final concentration) relative to control. Panel B: inhibition of [^{3}H]choline uptake by the addition of increasing concentrations of HC-3. Panel C: [^{3}H]choline uptake in the absence of potassium (Na^+-buffer), sodium (K^+-buffer), or both cations (NMG-buffer). Uptake is expressed relative to control parasites incubated in assay buffer. The data points represent mean values ± standard deviations of triplicate determinations from three independent experiments.

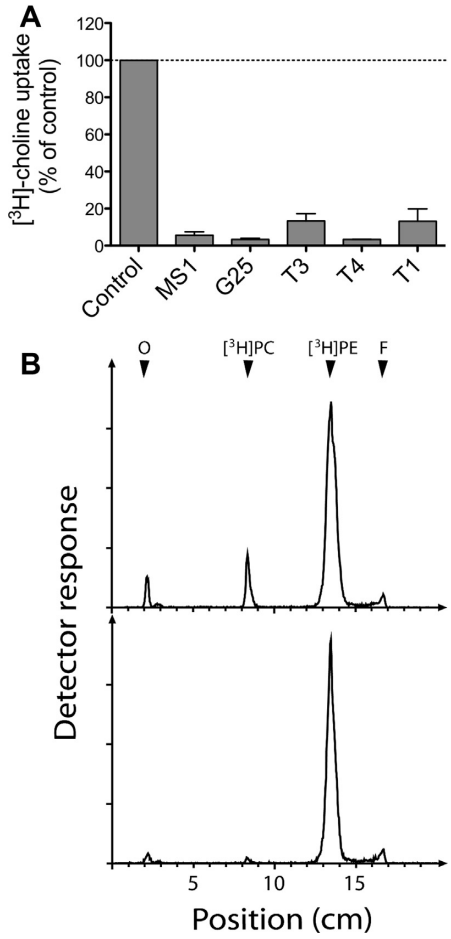

Fig. 4. Inhibition of choline uptake by choline analogs. Panel A: *T. brucei* procyclic forms were incubated in buffer containing 65 nM [^{3}H]choline and 1 μM unlabeled choline in the absence (control) or presence of choline analogs (5 μM, final concentrations) for 10 min and the amounts of cell-bound radioactivity were determined by scintillation counting. The data points represent mean values ± standard deviations of triplicate determinations from three independent experiments. Panel B: *T. brucei* procyclic forms (4×10^7 parasites in 5 ml culture medium) were incubated for 2 h with 2 μCi/ml [^{3}H]choline and 0.25 μCi/ml [^{3}H]ethanolamine (as control) in the absence (top panel) or presence (bottom panel) of 5 μM G25. Subsequently, lipids were extracted and analyzed by TLC, and radiolabeled products were detected by radioisotope scanning. The arrows indicate the migration of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) standards run on the same TLC plate. O, site of sample application; F, migration distance of solvent front.

T. brucei is not driven by a sodium or potassium gradient, but is dependent on mitochondrial energy production and/or is proton-driven.

3.3. Inhibition of choline transport by choline analogs

It has been shown that choline transport in *Plasmodium* and *Leishmania* can be inhibited by several choline analogs [7,14]. We now show that these compounds also effectively block [^{3}H]choline uptake in *T. brucei* procyclic forms. In the presence of the choline-derived analogs MS1, G25, T3, T4, and T1 (5 μM, final concentrations; the structures of the compounds are given in [7]), [^{3}H]choline uptake was inhibited by >80–95% compared to control values (Fig. 4A). Since the compounds have been shown to be toxic for *T. brucei* upon longer exposure [7], cell viability and transport activity during drug treatment was verified by incubating parasites in the presence of [^{3}H]inositol or [^{3}H]ethanolamine. We found that 30 min of drug exposure had no effect on [^{3}H]inositol or [^{3}H]ethanolamine uptake into *T. brucei* procyclic forms (results not shown). In addition, our results showed that while treatment of *T. brucei* procyclic forms with G25 completely prevented [^{3}H]PC formation (as a result of inhibition of [^{3}H]choline uptake; see Fig. 4A), the synthesis of [^{3}H]PE was not affected (Fig. 4B). Together, these results show that choline analogs specifically inhibit uptake of choline in *T. brucei* procyclic forms without altering transport of other lipid precursors, demonstrating that the drugs did not affect

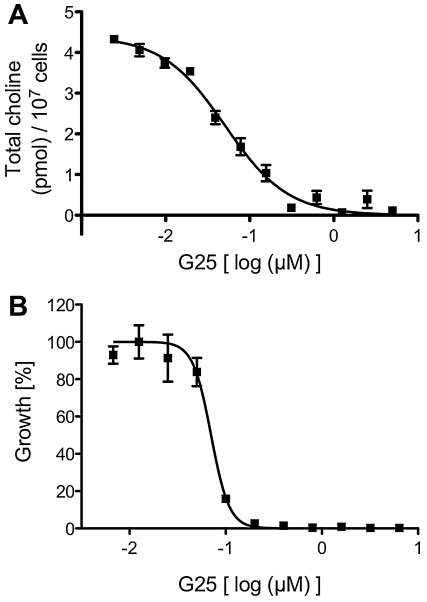

Fig. 5. Effect of G25 on choline uptake and parasite viability. Panel A: *T. brucei* bloodstream forms (5×10^6 parasites) were incubated in buffer containing 65 nM [^{3}H]choline and 1 μM unlabeled choline in the presence of increasing concentrations of G25 for 10 min and the amounts of cell-bound radioactivity were determined by scintillation counting. Data points represent mean values ± standard errors of triplicate determinations from one of two experiments. Panel B: susceptibility of *T. brucei* bloodstream forms to G25 was determined by Alamar blue assay as described in Section 2. Data points represent mean values ± standard deviations of triplicate determinations from three independent experiments.

parasite viability during the experimental time frame (10 min for [^{3}H]choline uptake studies and 2 h for determination of de novo phospholipid synthesis).

Incubation of *T. brucei* procyclic and bloodstream forms in buffer in the presence of 1 μM choline and increasing concentrations of G25 revealed IC_{50} values for choline transport inhibition of 37.5 ± 9.2 nM (not shown) and 54.0 ± 5.0 nM (Fig. 5A), respectively. Much higher concentrations of G25 were needed to inhibit choline uptake when parasites were incubated in the presence of choline in culture medium. For example, the addition of 1 μM G25, i.e. a value close to 20-fold the IC_{50} measured in buffer, to *T. brucei* procyclic and bloodstream forms in culture medium only inhibited choline uptake by 77% and 75%, respectively (mean values from two separate experiments). This difference can, at least in part, be attributed to the amounts of choline present in the culture media (28.6 μM and 7.2 μM in HMI-9 and SDM-79, respectively). In addition, in agreement with a previous report [7], we found that the EC_{50} value of G25 for growth inhibition of *T. brucei* bloodstream forms, as determined using Alamar blue assays, was in the low nanomolar range (70 ± 10 nM; Fig. 5B), i.e. at a concentration that has no effect on choline uptake in trypanosomes in culture medium. Thus, despite being a potent inhibitor of choline uptake, G25 may not exert its toxic effect in *T. brucei* by inhibition of choline transport.

3.4. Search for a putative T. brucei choline transporter

Our results showing that choline uptake in *T. brucei* is Na^+-independent and inhibited by HC-3 with an IC_{50} in the low micromolar range suggest that it may occur via CTL-mediated transport. A bioinformatics search aimed at de-orphanizing putative transporters of *T. brucei* identified significant similarity between a cluster of six *T. brucei* proteins and human CTL transporters as determined by HMM-HMM alignment [26] (Suppl. Fig. 1). Five of the genes, Tb927.10.6720, Tb927.10.6730, Tb927.10.6740, Tb927.10.6750 and Tb927.10.6760 (collectively referred to as Tb927.10.20-60), are highly similar and are arranged in tandem on chromosome 10, while the last gene, Tb927.10.530, shows less sequence similarity to Tb927.10.20-60 and clearly separates phylogenetically (Suppl. Fig. 1). To study a possible involvement of the gene products in choline uptake in *T. brucei*, expression of Tb927.10.20-60 and Tb927.10.530 was down-regulated by RNAi in procyclic forms. Because of the sequence homology among the members of the Tb927.10.20-60 gene cluster, we designed a single construct to down-regulate transcript levels of all five genes. Northern blot analysis showed that after induction by tetracycline for 3 days, Tb927.10.20-60 mRNAs were clearly decreased compared to control levels (Suppl. Fig. 2A). However, parasite growth was not affected during 12 days of culture in the presence of tetracycline (Fig. 2A) and uptake of [^{3}H]choline was unchanged compared to control uninduced cells (results not shown). Similarly, no difference in growth and [^{3}H]choline uptake was observed in *T. brucei* bloodstream forms after down-regulation of Tb927.10.530 mRNA levels by 85% (Suppl. Fig. 2B, and results not shown).

Furthermore, we expressed Tb927.10.6740, Tb927.10.6760, and Tb927.10.530 in an *hnm1* yeast mutant lacking the gene encoding the *S. cerevisiae* choline transporter, to study if the *T. brucei* gene products could functionally complement Hnm1p. The results showed that expression of the *T. brucei* genes in *S. cerevisiae* had no effect on uptake of [^{3}H]choline, while expression of *HNM1* restored [^{3}H]choline transport (positive control) (Suppl. Fig. 3).

Finally, Tb927.10.6750 and Tb927.10.530 were expressed separately in *Xenopus laevis* oocytes to study choline transport in a well-established expression system for carriers [29]. However, using two-electrode voltage clamp measurements, no electrogenic transport activity was detected after injection of the corresponding mRNAs (results not shown). In addition, no uptake of [^{3}H]choline was measured using tracer flux experiments. Together, these results indicate that the *T. brucei* genes identified by sequence comparison with human CTL transporters unlikely encode major choline uptake systems in *T. brucei*.

4. Discussion

Early studies using *T. brucei* parasites isolated from the blood of infected rats showed that in the presence of *lyso*-PC, bloodstream form trypanosomes take up little choline from the environment in vitro [4]. More recently, direct measurements using radioactive substrates failed to detect choline uptake in *T. brucei* bloodstream forms [7,8], suggesting that *T. brucei* parasites, which are considered choline auxotrophs [1], lack a choline transport system and meet their demand for choline by uptake of *lyso*-PC.

In contrast, we now show that *T. brucei* bloodstream and procyclic forms in culture readily take up choline. Using choline concentrations of 0.12–62.5 μM, uptake of choline occurred temperature-dependent and linear over at least 9 min and was saturable, with V_{max} values of 5.7 and 8.1 pmol/min x 10^7 for bloodstream and procyclic forms, respectively. At present, it is unclear why choline uptake was not detected in a previous study [7]. Possible reasons may include short incubation times (1 min), low concentrations of choline (250 nM), and the use of bloodstream form parasites isolated from infected rats.

Our observations that choline transport in trypanosomes (i) follows Michaelis–Menten kinetics, with a K_m in the low micromolar range, (ii) can be inhibited by the specific choline transport inhibitor, HC-3, and (iii) is sodium- and potassium-independent, indicates that it is mediated by an intermediate-affinity transport

system. Similar observations have been reported in *Plasmodium* [14] and *Leishmania* [13], suggesting that protozoan parasites may rely on closely related transporters for choline uptake. However, in none of these parasites, a choline transporter has been identified. Unfortunately, although we have identified genes encoding candidate CTL proteins using a bioinformatics approach, neither down-regulation of their expression in *T. brucei* bloodstream and procyclic forms, nor their expression in *Xenopus* oocytes or *S. cerevisiae* provided evidence of their involvement in choline transport.

It has previously been shown that PC synthesis via the CDP-choline branch of the Kennedy pathway is essential for growth of *T. brucei* bloodstream [1] and procyclic forms [6]. This observation has been difficult to explain since trypanosomes lacked a transport system for uptake of the precursor of the pathway, choline. Our findings now resolve this conundrum by showing that choline taken up from the environment is readily used for de novo PC synthesis in *T. brucei* parasites in culture. Nevertheless, in the blood of an infected human, the situation may be different as the plasma concentration of *lyso*-PC is up to 10-fold higher than that of choline [30–32], suggesting that trypanosomes may meet their demand for precursors for PC synthesis primarily by uptake of *lyso*-PC.

Interestingly, our results show that choline transport in *T. brucei* is inhibited by a set of synthetic choline analogs. These compounds have previously been reported to block choline uptake in *Plasmodium* and *Leishmania* [7,14]. In addition, they are highly toxic for these parasites in culture, with EC_{50} values in the nanomolar range in *Plasmodium* [15,33] and micromolar range in *Leishmania* [7]. Similarly, we found that these choline analogs also inhibit proliferation of *T. brucei* bloodstream (in agreement with a previous report [7]) and procyclic forms. However, considering the IC_{50} and EC_{50} values of G25 in *T. brucei* bloodstream forms, it is unclear if the mode of action is due to inhibition of choline uptake. In addition, at present it is unknown how these drugs are taken up by protozoan parasites.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2013.05.007.

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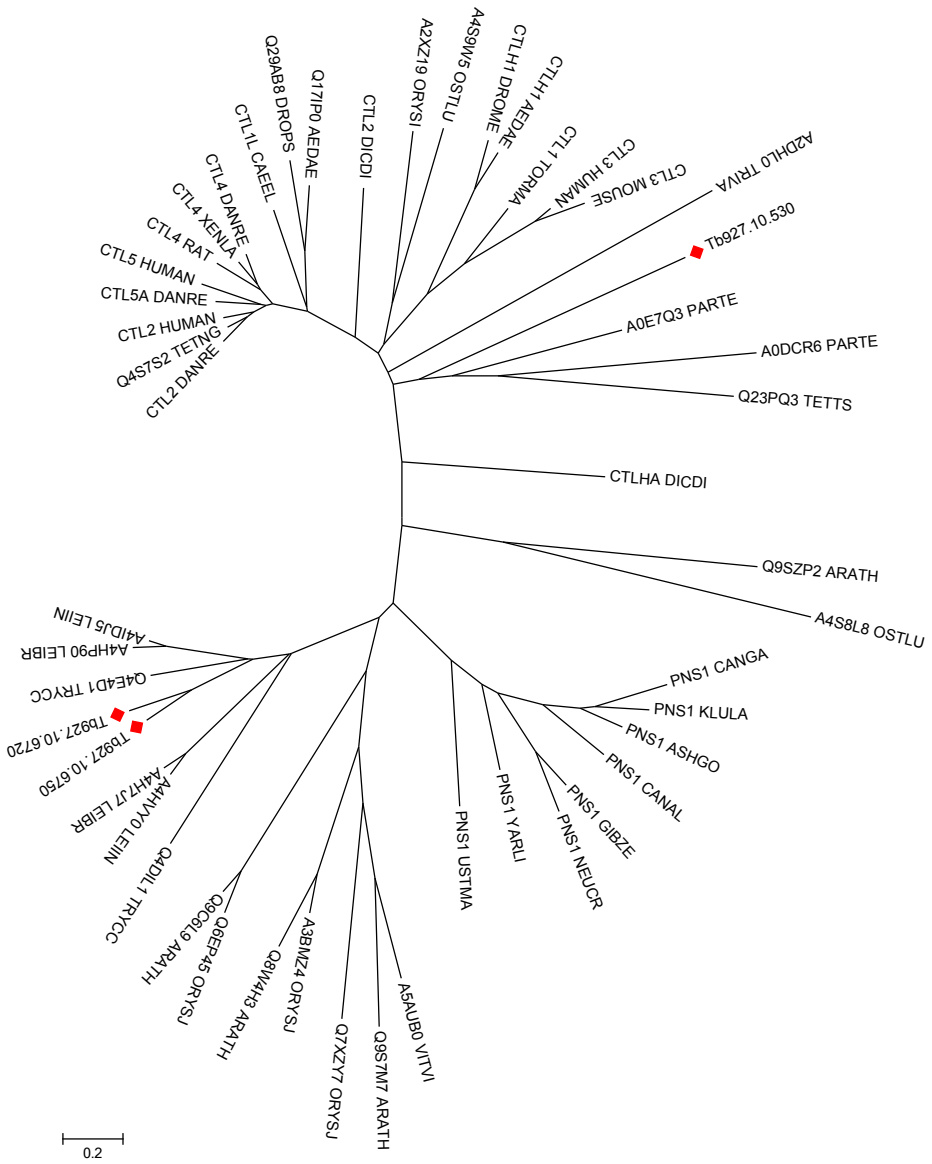
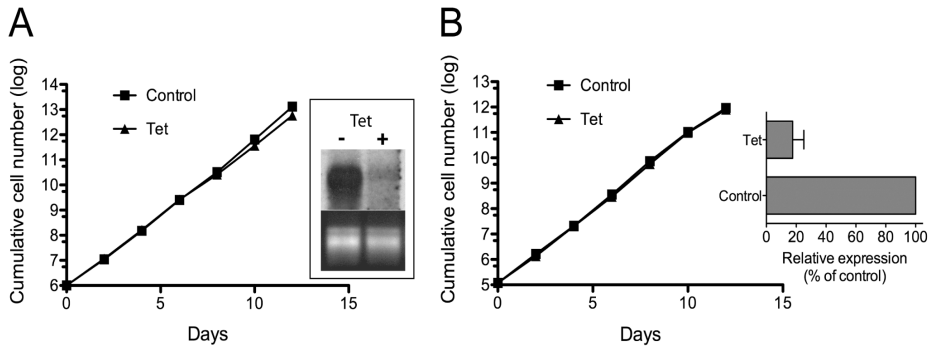
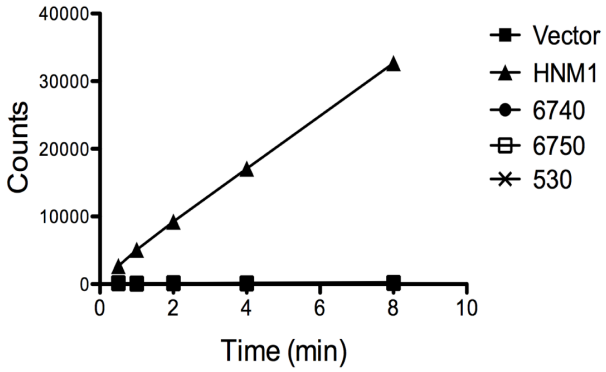
Supplementary Figures



Suppl. Fig. 1: Evolutionary relationships of predicted choline transporter domains. The tree was built from a redundancy reduced set of putative choline transporters of the seed alignment file of Pfam profile PF04515, using the Neighbor-Joining method and JTT distance metric with the program MEGA5. The three sequences from *T. brucei* are marked with red diamonds. Gene IDs are from UniProt. Units are the number of amino acid substitutions per site.



Suppl. Fig. 2: RNAi-mediated down-regulation of candidate choline transporters. *T. brucei* parasites harboring RNAi plasmids to down-regulate expression of Tb927.10.20-60 (panel A, procyclic forms) or Tb927.10.530 (panel B, bloodstream forms) were grown in the absence (Control) or presence (Tet) of tetracycline for 12 days. Parasite densities were determined every second day and are represented as cumulative cell numbers. The inset in panel A shows depletion of Tb927.10.20-60 mRNA by Northern blotting in parasites cultured for 2 days in the absence (-) or presence (+) of tetracycline (Tet) (upper panel), with rRNA as loading control (lower panel). The inset in panel B shows depletion of Tb927.10.530 mRNA by quantitative PCR in non-induced (control) and induced (Tet) parasites using 5'-TTCAAGGGAATCGGTGAGAC-3' as forward and 5-ACCACAGGAAGGCAAACAAC-3' as reverse primers (selected using Primer3plus software [1]). Telomerase reverse transcriptase (Tb927.01.1950) was used as a reference mRNA using primers described elsewhere [2]. The reaction was performed with 3 different dilutions of cDNA and efficiency was higher than 93%. Data points represent mean values ± SEM of triplicate determinations.



Suppl. Fig. 3: Choline uptake by mutant yeast transfected with candidate *T. brucei* choline transporters. Choline transporter-deficient yeast cells were transfected with plasmids containing Tb927.10.6740 (6740), Tb927.10.6750 (6750), Tb927.10.530 (530), or yeast choline transporter (HNM1) as positive control; the empty vector (Vector) was used as negative control. Choline uptake was determined by incubating cells in the presence of [^{3}H]choline (10 nM, final concentration) for 8 min and cell-bound radioactivity was measured by scintillation counting.

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2.2 An atypical mitochondrial carrier that mediates drug action in Trypanosoma brucei

(work in progress)

Author contributions:

G. Schumann and I. Roditi developed the RNAi library used in this study and provided technical input on the screening;

The metabolomics analyses were done in M. Barrett's lab;

H. Vial provided the choline analogs;

All experimental data and figures on this manuscript were generated by myself.

An Atypical Mitochondrial Carrier That Mediates Drug Action in *Trypanosoma brucei*

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Introduction

African trypanosomes are vector-borne protozoans that cause serious public health problems and uncountable economical losses in sub-Saharan African countries. The current chemotherapy against *Trypanosoma brucei*, the causative agent of human African trypanosomiasis, or sleeping sickness, and a related cattle disease, called Nagana, is based on repetitive injections of drugs with strong side effects. Treatment of Nagana is commonly based on carcinogenic drugs like ethidium bromide and isometamidium chloride, and also on suramin and diminazene aceturate, both with considerable toxicities to cattle [1]. In the case of human African trypanosomiasis, pentamidine and suramin are widely used during the first stage of the disease, when parasites are still present in the hemolymphatic tissues. The second stage of sleeping sickness, which is characterized by parasite invasion of the central nervous system, is treated with melarsoprol or a combination of nifurtimox/eflornithine [2]. Melarsoprol, the only drug against both forms of sleeping sickness, i.e. caused by *T. b. gambiense* or *T. b. rhodesiense*, is highly toxic causing encephalopathies in 5% of cases. In addition, there are well-known examples of cross-resistance in the field [3,4], demonstrating that selection of resistant strains is a real threat to human health in endemic regions. Hence, more efforts to develop new drugs and optimize treatment for sleeping sickness are needed.

T. brucei and other protozoan parasites, by definition, acquire nutrients and building blocks for rapid cell proliferation from their mammalian or insect hosts. However, recent reports have shown that trypanosomatids not only acquire lipids for membrane formation from the environment, but are also capable of *de novo* synthesis of all major membrane lipid classes [5]. The most abundant phospholipid class in *T. brucei* parasites is phosphatidylcholine (PC) [6], which can be generated by acylation of lyso-PC taken up from the host [7]. Alternatively, PC can be produced from host-derived choline [8] by sequential action of three enzymes via the CDP-choline pathway (reviewed in [5]). This pathway is essential for survival of *T. brucei* parasites in culture [5].

PC is also the most abundant phospholipid class in the malaria parasite, *Plasmodium spp.* (reviewed in [9]). Its synthesis can occur via multiple routes, including the CDP-choline pathway (reviewed in [10]). The inability to knock-out individual genes involved in this pathway suggests that PC formation via CDP-choline is essential in Plasmodium [11]. In addition, the uptake of the substrate for this pathway, choline, can be inhibited by a set of choline analogs, which have been found to be toxic for malaria parasites [12,13,14]. However, at present it is unclear if these drugs affect parasite viability due to their potency to prevent choline uptake or their inhibitory effect on enzymes of the CDP-choline pathway [15,16,17,18]. Structural refinements of the drugs has lead to the development of third and fourth generation compounds, one of which, named T3, is currently in clinical trials to treat malaria infections [18]. More recently, a subset of these compounds has also been shown to be

toxic for *T. brucei* and Leishmania parasites [19]. Their mode of action is unclear: although they effectively inhibit choline uptake and, thus, de novo PC formation in *T. brucei* [8], they may kill trypanosomes by affecting mitochondrial structure and function [8,19].

In the present study, we used three of the leading choline analogs, two bis-tiazolium salts, T3 and T4, and a bis-quartenary ammonium salt, G25, to elucidate the site and mode of action of the compounds. For this, we screened an RNAi library previously established in *T. brucei* bloodstream forms [20] to identify genes conferring parasite resistance towards the drugs. Interestingly, we found that treatment of *T. brucei* bloodstream forms with these drugs selected parasite populations in which the expression of a gene encoding a mitochondrial carrier was down-regulated.

Materials and methods

All reagents were of analytical grade and purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (Buchs, Switzerland) or ICN Biomedicals (Tägerig, Switzerland). Antibiotics and fetal bovine serum (FBS) were obtained from Invitrogen (Basel, Switzerland). Dialyzed fetal calf serum (FCS) was obtained from BioConcept Amimed (Allschwil, Switzerland). DNA polymerases were purchased from Promega (Madison, USA). Primers and sequencing services were from Microsynth AG (Balgach, Switzerland). All restriction enzymes were purchased from Fermentas (Nunningen, Switzerland). [α-^{32}P]-dCTP (3000 Ci/mmol) was from PerkinElmer Life Sciences (Schwerzenbach, Switzerland) and Kodak MXB and BioMax MS films were from Kodak SA (Lausanne, Switzerland).

Trypanosomes and culture conditions

T. brucei bloodstream forms co-expressing T7 RNA polymerase and a tetracycline repressor [21] were cultured at 37 °C in HMI-9 containing 10% (v/v) heat-inactivated FBS. Derived clones to down-regulate or over-express TbMCP14 were cultured in the presence of 2.5 µg/ml phleomycin or 0.1 µg/ml puromycin, respectively. TbMCP14 knock-out parasites were cultured in the presence of 5 µg/ml hygromycin and 2.5 µg/ml blasticidin. *T. brucei* 29-13 procyclic forms [21] were cultured at 27 °C in SDM-79 containing 10% (v/v) heat-inactivated FBS, in the presence of 25 µg/ml hygromycin, and 15 µg/ml G418. The derived clones containing different double-stranded RNA constructs against TbMCP14 (Tb927.10.13120) were cultured in the presence of an additional 2 µg/ml puromycin. TbMCP14 conditional knock-out procyclic forms were cultivated in the presence of 5 µg/ml blasticidin, 0.2 µg/ml phleomycin and 2 µg/ml puromycin, and 1 µg/ml tetracycline to maintain expression of the ectopic copy of TbMCP14. Growth of *T. brucei* procyclic forms in glucose-depleted

medium was studied using SDM-80 [22], supplemented with 9% dialyzed (10'000 molecular weight cut-off) and heat-inactivated FCS and 1% non-dialyzed and heat-inactivated FBS.

Drug sensitivity assays and screening of RNAi library

Susceptibility of *T. brucei* bloodstream forms to G25, T3 and T4 was assessed by Alamar blue assays [23]. Briefly, serial dilutions of G25, T3 or T4 starting at 10, 250 or 10 µM (from 10 mM stock solutions), respectively, were prepared in HMI-9 containing 10% (v/v) FBS in 96-well plates (100 µl final volume). Parasites were added to a final density of $1x10^4$ cells/ml. After incubation for 70 h at 37 °C, 10 µl of Alamar blue solution (12.5 mg of resazurin in 100 ml PBS, composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2) was added to all wells and incubation was continued for another 2 h at 37 °C. Fluorescence was measured using a spectromax GEMINI plate reader at 544 nm excitation, 590 nm emission and 570 nm cutoff. Drug screening using an RNAi library constructed in *T. brucei* bloodstream forms was performed as described before [20]. Briefly, a frozen stabilate of $3x10^6$ parasites was thawed in 30 ml HMI-9 supplemented with 10% FBS and daily diluted to the same density ($3x10^6$ cells/30 ml). After three passages, RNAi was induced by addition of tetracycline to the culture. After three days, the parasite culture was split in flasks containing 10^6 cells/10 ml medium. Drug concentrations determined to kill 98% of parasites (EC_{98}) by Alamar blue assays were used for selection of resistant parasites. EC_{98} concentrations of G25, T3 or T4 were added to the culture flasks, which were incubated until resistant clones started growing. Finally, genomic DNA of resistant parasites was extracted and the putative DNA fragments conferring resistance amplified by PCR, cloned into a PCRII-TOPO vector (Invitrogen) and identified by DNA sequencing.

RNAi-mediated gene silencing, over-expression and generation of knock-out parasites

Two tetracycline-inducible vectors (derivatives of pLew100 [21]), both harboring an inducible stem loop, were used to down-regulate TbMCP14. A first plasmid, in which the stem loop is under control of the PARP promoter [24], was used to transfect procyclic forms, whereas a second plasmid, which is regulated by an rRNA promoter [25], was used to transfect bloodstream forms. TbMCP14 gene fragments were cloned into the vectors using PCR products obtained with primers 1 and 2 (Table S1), resulting in plasmids pJPM14pc and pJPM14bs.

For over-expression in procyclic and bloodstream form trypanosomes, the TbMCP14 open reading frame (amplified using primers 3 and 4, Table S1) was inserted into an expression vector

based on pLew100 [21,26], containing a C-terminal extension encoding cMyc to allow tetracycline-inducible expression of cMyc-tagged TbMCP14, resulting in plasmid pJPM14O. The same strategy was used to induce over-expression of the truncated version of TbMCP14 except that a shorter open reading frame (amplified using primers 5 and 4, Table S1) was inserted in the vector.

Procyclic form TbMCP14 conditional knock-out parasites were generated stepwise by i) replacing one of the endogenous alleles with a blasticidin resistance gene by homologous recombination, ii) inserting an ectopic copy of TbMCP14, C-terminally tagged with cMyc, and iii) replacing the remaining endogenous allele of TbMCP14 by a phleomycin resistance gene. The corresponding vectors were generated as follows; first, a fragment of 400 nt from the 5'-UTR region of TbMCP14 was amplified using primer 6, having an XhoI restriction site, and primer 7, having a HindIII restriction site (Table S1). Second, a fragment of 458 nt from the 3'-UTR region of TbMCP14 was amplified using primers 8 and 9 (Table S1). Subsequently, the two fragments were inserted into vector pKO^{blast} [27], which contains the procyclin EP1-EP2 intergenic region, a blasticidin resistance gene, and the tubulin intergenic region, resulting in vector $pJPM14KO^{blast}$. The blasticidin resistance gene was then replaced by a phleomycin resistance gene using the flanking restriction sites AscI and PacI, resulting in vector $pJPM14KO^{phleo}$.

Plasmid extraction was performed using Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before transfection of *T. brucei* parasites, pLew-based plasmids were linearized with *Not*I while pKO plasmids were digested with XhoI/NotI to release linear fragments containing the respective resistance genes flanked by sequences for homologous recombination with TbMCP14.

Stable transfections of trypanosomes

T. brucei procyclic and bloodstream forms were harvested at mid-log phase, washed once in buffer (132 mM NaCl, 8 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM magnesium acetate, 0.09 mM calcium acetate, pH 7.0) and resuspended in fresh buffer at a density of $8x10^7$ cells/ml. Subsequently, 440 µl of parasite suspension were mixed with 10-15 µg of digested plasmids and transferred to a 0.2-cm pulse cuvette (Bio-Rad). Electroporation was conducted with a BTX Electroporation 600 system (Axon Lab, Baden, Switzerland) with one pulse (1.5 kV charging voltage, 2.5 kV resistance, 25 microfarads capacitance timing, and 186 resistance timing). Cells were immediately inoculated in 10 ml of procyclic or bloodstream form medium. Dilutions were plated into 24-well plates and after 24 h selected for antibiotic resistance. Clones were obtained by limiting dilution.

Northern blot analysis

Total RNA was isolated using the SV Total RNA Isolation System (Promega), following the manufacturer's instructions. cDNA was synthesized from total RNA (0.1-0.5 µg) using SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR was performed using Applied Biosystems ViiA7 real-time PCR system. The reaction mixture consisted of 1x Fast SYBR Green master mix (Applied Biosystems, Switzerland), 0.45 µM forward and reverse primers (primers 10 and 11, respectively, Table S1). Telomerase reverse transcriptase mRNA was amplified using primers described in [28] and was used as endogenous control for qPCR analyses. For Northern blotting, total RNA (10-15 µg) was separated on formaldehyde-agarose gels (1% agarose, 2% formaldehyde in 20 mM Mops, containing 8 mM sodium acetate and 1 mM EDTA, pH 7.0) and transferred to Amersham Hybond-N^+ nylon membranes (GE Healthcare, Buckinghamshire, UK). The PCR products used to construct the RNAi stem loop vectors served as templates to make the [^{32}P]-labeled probes by random priming, using Prime-a-Gene Labeling System (Promega). Hybridization was performed overnight at 60 °C in hybridization buffer containing 7% (w/v) SDS, 1 mM EDTA, 0.5 M Na_2HPO_4, 24 mM H_3PO_4, pH 7.2, and the membrane was analyzed by autoradiography using BioMax MS film and a TransScreen-HE intensifying screen. Ribosomal RNA was visualized on the same formaldehyde-agarose gel by ethidium bromide staining to control for equal loading.

Fluorescence microscopy

Parasites (10^6) were allowed to adhere to a microscopy slide for 10 min and then fixed with 4% paraformaldehyde in PBS. Subsequently, parasites were washed with PBS and permeabilized with 0.2% (w/v) Triton X-100 in PBS. After incubation in PBS containing 2% bovine serum albumin (blocking buffer) for 30 min, primary antibody in blocking solution was added for 45 min. Antibodies used were mouse monoclonal anti-cMyc 9E10 (Santa Cruz Biotechnology, Heidelberg, Germany) and rabbit anti-VDAC antiserum at dilutions of 1:250 and 1:1000, respectively. After washing with PBS, the corresponding secondary fluorophore-conjugated antibodies, goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 (Invitrogen), respectively, at dilutions of 1:100 in blocking solution, were added for 45 min. Free antibody was removed by washing with PBS and cells were mounted with Vectashield containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The slides were analyzed using a Leica SP2 microscope equipped with a 100× oil objective. Photographs were acquired with Leica LAS AF Version 2.1.0 software (Leica Microsystems).

Flow cytometry

T. brucei bloodstream forms were incubated in the presence of 80-350 nM G25 for 24 or 48 h. Aliquots of 0.5 ml were taken to measure mitochondrial membrane potential ($\Delta\Psi_m$) and cell permeability by propidium iodide (PI) staining. The $\Delta\Psi_m$ was measured by adding 25 nM or 125 nM tetramethylrhodamine ethyl ester (TMRE) to bloodstream or procyclic form cultures, respectively. After 30 min of incubation at 37 °C (bloodstream forms) or 27 °C (procyclic forms), parasites were washed with and resuspended in PBS, and immediately analyzed by flow cytometry (FACScan BD, equipped with Cytek solid state laser) using the FL2-channel detector. The geometrical mean values of 10'000 gated events were normalized according to the control samples. For evaluation of cell permeability, 10 µg/ml of PI was added to the respective culture medium and incubated for 10 min at 37 °C or 27 °C (for bloodstream or procyclic form experiments, respectively), protected from light. Subsequently, 0.5 ml of parasite culture was transferred to FACS tubes and fluorescence was measured using FL3-channel detector. Ten thousand gated events were separated into two areas - according to fluorescence intensity - as follows: the fluorescence intensity of a sample containing digitonin-permeabilized parasites was measured and referred to as PI-positive (PI+). Fluorescence intensities lower than this value were considered PI-negative (PI-).

Results

Screening of RNAi library and identification of TbMCP14

Recently, an inducible RNAi library has been established in *T. brucei* bloodstream forms, which allows an unbiased approach to identify genes involved in drug uptake or action [20]. We have used this library to elucidate the mode and site of action of a set of choline analogs known to be toxic for parasitic protozoa, including *T. brucei* [14,16,19]. In a first step, we determined the concentrations of G25, T3 and T4 required to kill 98% of *T. brucei* bloodstream forms after 3 days of culture using Alamar blue assays (EC_{98}) [20]. The obtained values are shown in Fig. 1A, together with the EC_{50} values to allow comparison with previously published data [19]. The EC_{98} concentrations were subsequently used to treat separate trypanosome cultures with G25, T3 and T4, following induction of RNAi for 60 h with tetracycline (Fig. 1B). After 8 days, parasites cultured in the absence of tetracycline were dead, while resistant cells started to proliferate. After another 3 days of culture, during which time parasites were kept in fresh medium to allow optimal growth, DNA was extracted and inserts potentially conferring resistance towards the drugs were amplified using specific primers [20]. Interestingly, the individual screens using G25, T3 and T4 all resulted in selection of trypanosomes bearing RNAi inserts partially covering the gene encoding putative mitochondrial

carrier protein 14 (TbMCP14; Tb927.10.13120), a member of a large family of mitochondrial carriers [29]. Screening with G25 and T3 selected parasites bearing the same RNAi insert, while T4 selected parasites harboring a different TbMCP14 RNAi sequence (Fig. 1C, bottom, indicated in color). Tb927.10.13120 has been annotated to comprise of an ORF of 1035 bp (TriTrypDB, GeneDB) and, by transient transfection using a C-terminally tagged protein, has previously been shown to localize to the mitochondrion [29]. However, recent results from transcriptome analyses suggested that a second potential start codon 171 bp upstream of the annotated ATG may exist [30,31]. Using RT-PCR involving a splice leader primer (primer 12 Table S1) and TbMCP14 reverse primers (primers 2 and 4, Table S1), we amplified transcripts from *T. brucei* bloodstream and procyclic forms consistent with Tb927.10.13120 encoding an mRNA of 1206 bp (Fig. 1C, right panel) with a predicted full-length protein of 401 amino acids.

TbMCP14 is closely linked to drug action

To verify the involvement of TbMCP14 in drug resistance towards choline analogs, expression of Tb927.10.13120 was down-regulated by inducible RNAi in *T. brucei* bloodstream forms. Addition of tetracycline to parasites in culture showed no growth phenotype (Fig. 2A), despite a reduction in Tb927.10.13120 transcript level by >70% (result from two independent experiments) as determined by qPCR. The non-essentiality of Tb927.10.13120 as assessed by RNAi was not surprising since trypanosomes selected from the RNAi library were viable after 11 days of RNAi induction (see above). However, as expected, treatment of parasites after down-regulation of Tb927.10.13120 expression showed increased resistance towards G25, T3, and T4 (Fig. 2B-D). In Alamar blue assays, the EC_{50} values of TbMCP14-depleted bloodstream form parasites for the drugs increased an average of 7-fold compared to uninduced cells. Subsequently, Tb927.10.13120 expression was also down-regulated using RNAi in *T. brucei* procyclic forms. Prolonged incubation of parasites in the presence of tetracycline showed a decrease in Tb927.10.13120 mRNA levels and a slight growth phenotype (Fig. 3A). In agreement with the results obtained in bloodstream forms (Fig. 2B-D), RNAi against Tb927.10.13120 conferred increased resistance (approximately 3-fold) of procyclic form parasites towards T3 (Fig. 3B).

To further confirm the involvement of TbMCP14 in drug action, we over-expressed a tetracycline-inducible ectopic copy of cMyc-tagged Tb927.10.13120 in procyclic and bloodstream form trypanosomes. Analysis by SDS-PAGE and immunoblotting showed that cMyc-TbMCP14 was expressed in the presence, but not in the absence, of tetracycline as a 48 kDa protein, and that expression of full-length cMyc-TbMCP14 conferred increased susceptibility (>10-fold) to parasites against T3 (Fig. 3C). In contrast, no effect on sensitivity towards T3 was observed in parasites over-

expressing a truncated form of TbMCP14, which migrated after SDS-PAGE and immunoblotting with an apparent molecular mass of 42 kDa (Fig. 3D). These results clearly demonstrate that the tagged version of the full-length protein is functional.

Additionally, viability of *T. brucei* bloodstream forms over-expressing c-Myc-TbMCP14 (full-length) cultured in the presence of various concentrations of G25 was investigated by propidium iodide (PI) staining. While dead parasites are permeable to PI, resulting in its intercalation into DNA with subsequent fluorescence emission (PI positive), living parasites are impermeable to PI and show no emission of fluorescence. The results show that over-expression of TbMCP14 slightly increased toxicity of G25 (Figure 4, top right), whereas its down-regulation had a protective effect on parasite survival in the presence of the drug (Figure 4, bottom right). In addition, the data show that G25 is only toxic for *T. brucei* bloodstream forms after prolonged incubation, i.e. 72 h (Fig. 4, top left, green line). Taken together, the results demonstrate that TbMCP14 expression is closely linked to the action of the choline analogs, G25, T3 and T4.

Localization of TbMCP14

In a previous report, the truncated version of TbMCP14 has been expressed as cMyc-tagged version and was found to localize in the mitochondrion [29]. We now show by immunofluorescence microscopy that also full-length TbMCP14 localizes to the mitochondrion, co-localizing with the mitochondrial marker protein, voltage-dependent anion channel (VDAC) (Fig. 5), and demonstrating that the N-terminal 57 amino acids of full-length TbMCP14 are not essential for correct targeting of the protein to the mitochondrion.

TbMCP14 mediates the effects of choline analogs on mitochondrial membrane potential ($\Delta\Psi_m$)

It has been suggested that the trypanocidal activity of choline analogs may occur via modulation of mitochondrial membrane potential ($\Delta\Psi_m$) [19]. We revisited this proposal by measuring drug-induced changes in $\Delta\Psi_m$ in *T. brucei* bloodstream forms, in which TbMCP14 was over-expressed or down-regulated. Using the mitochondrial dye, tetramethylrhodamine ethyl ester (TMRE), and flow cytometry, we found that G25 induced a decrease in $\Delta\Psi_m$ following treatment of parasites for 24 h with increasing concentrations of G25 (Fig. 6A). This drug-induced decrease in $\Delta\Psi_m$ was absent in parasites after RNAi-mediated down-regulation of TbMCP14 (Fig. 6A). Conversely, sensitivity of $\Delta\Psi_m$ to G25 was increased in parasites after over-expression of TbMCP14 (Fig. 6B). In control experiments, parasite viability during drug treatment was measured by incorporation of PI

and flow cytometry analysis. We found no difference in propidium iodide staining between parasites before and after incubation for 24 h in the presence of G25, ruling out the possibility that the observed decrease in $\Delta\Psi_m$ was the result of parasite death, rather than drug action (Suppl. Fig. S1). Together, these results demonstrate that TbMCP14 is tightly involved in drug-induced changes of $\Delta\Psi_m$ and that they are an early event during G25 action.

Generation of TbMCP14 knock-out parasites

To further study the importance of TbMCP14 in *T. brucei* viability, we generated bloodstream and procyclic form Tb927.10.13120 null mutants. Since RNAi against TbMCP14 in bloodstream forms showed no growth phenotype, we attempted to generate straight knock-out parasites by sequentially deleting the two endogenous Tb927.10.13120 alleles. Successful replacement of the first and second alleles by blasticidin resistance and phleomycin resistance genes, respectively, was verified by PCR (Fig. 7A). The resulting TbMCP14 null bloodstream forms were viable, however, they showed reduced growth in culture compared to the parental cell line and the single allele knock-out mutant (Fig. 7B). The cell doubling time of the null mutant was calculated to be 12.5 h, compared to 6 h of the parental strain. Together, the results show that TbMCP14 is essential for normal growth of *T. brucei* bloodstream forms in culture, but non-essential for viability under these conditions.

Because *T. brucei* procyclic forms showed slightly reduced growth in culture after RNAi against TbMCP14 (Fig. 3A), we followed a different strategy to obtain procyclic form TbMCP14 null mutants by introducing a tetracycline-inducible ectopic copy of Tb927.10.13120 before knocking out the second allele. To be able to monitor expression of ectopic TbMCP14, the gene was extended with a sequence encoding cMyc at its 3' end. Again, successful deletion of the two endogenous alleles was verified by PCR (Fig. 7A). Resulting parasites cultured in the presence of tetracycline, i.e. expressing cMyc-tagged ectopic TbMCP14, grew normally in standard growth medium (Fig. 7C). In contrast, after removal of tetracycline from the culture medium, parasites showed reduced growth after 6 days of culture and eventually stopped proliferating (Fig. 7C). SDS-PAGE and immunoblotting revealed that cMyc-tagged TbMCP14 was expressed in parasites cultured in the presence of tetracycline, but disappeared during ablation of TbMCP14 expression after removal of tetracycline (Fig. 7D). Together, the results show that TbMCP14 is essential for growth of *T. brucei* procyclic forms in culture and that cMyc-tagged TbMCP14 is functional.

TbMCP14 is involved in energy metabolism

Our initial approach to elucidate the physiological function of TbMCP14 was to identify changes in small metabolites in *T. brucei* procyclic forms over-expressing or down-regulating

TbMCP14 by running an untargeted metabolomic analysis [32]. The approach revealed that the proline metabolite, pyrroline-5-carboxylate, was 4-fold increased in parasites over-expressing TbMCP14, suggesting that TbMCP14 may play a role in amino acid metabolism. Thus, we investigated if down-regulation of TbMCP14 may show a more dramatic effect when trypanosomes are grown in glucose-depleted medium, SDM80, in which procyclic forms are known to increase the consumption of proline for energy production by >6 times [22]. The results showed that in SDM80, RNAi against TbMCP14 caused a much stronger growth defect compared to when parasites were grown in SDM79, resulting in cell death after 10 days of induction (Fig. 8A, compare with Fig. 3A). When the medium was supplemented with 5.5 mM glucose the effect of down-regulation was delayed by about three days (Fig. 8A). Together, these results indicate that TbMCP14 indeed may be involved in metabolism of amino acids for energy production. In line with this interpretation, we found that depletion of TbMCP14 in conditional knock-out parasites cultured in the absence of glucose clearly reduced cell growth (Fig. 8B), whereas expression levels of TbMCP14 had no effect on growth of parasites cultured in the presence of glucose (Fig. 8C).

Discussion

Several studies have shown that choline analogs are potent inhibitors of choline uptake and affect PC metabolism in protozoa [12,13,19]. However, more recent results [19], including our own involving pulse-chase experiments using labeled choline in presence of G25 in *T. brucei* procyclic forms [8], indicated that inhibition of phospholipid synthesis may not be the main target of G25 in trypanosomes. Instead, results from these studies suggested that choline analogs may affect mitochondrial function and, as a result, lead to parasite death. However, a clear mechanism on the uptake and site of action of the drugs was not proposed. We now demonstrate that toxicity of the choline analogs G25, T4 and T3, is mediated by the mitochondrial carrier, TbMCP14. Evidence for the involvement of TbMCP14 in drug action is several fold: First, TbMCP14 was identified by an unbiased screen using a RNAi library in *T. brucei* bloodstream forms. Its down-regulation resulted in selection of parasite clones showing increased resistance towards all three drugs used in the screen. Second, depletion of TbMCP14 by transcript-specific RNAi in both bloodstream and procyclic forms increased resistance of parasites towards the drugs by 8- and 3-fold, respectively. Third, over-expression of a tagged form of TbMCP14 in procyclic forms resulted in hypersensitivity towards T3. Fourth, down-regulation of TbMCP14 protected procyclic form mitochondria from drug-induced decrease in mitochondrial membrane potential, whereas over-expression caused increased sensitivity.

Additionally, the mode of action suggested for T3, T4 and G25 can also be extended to two additional choline-derived analogs, MS1 and M38 (see [19] for the structures), because a similar increase in the EC_{50} value was also observed after down-regulation of TbMCP14 in bloodstream forms (not shown). In contrast, no difference in resistance was observed when TbMCP14-depleted parasites were treated with the mono-cationic salt T1 (result not shown), indicating that T1 has a different mode of action.

It still not clear how choline analogs cross the plasma membrane of parasites. Previous studies have postulated that choline analogs are carried by the choline carrier of plasmodium [12,18]. Using radiolabeled T16, an analog of G25, Biagini *et al.,* studied the uptake kinetics into saponin-freed plasmodium and found similar properties with choline uptake kinetics. In addition, experiments using radiolabeled T3 and choline indicated reciprocal inhibition with T3 showing higher potency on choline uptake inhibition then otherwise [18].

The mitochondrial carrier family (MCF) consists of a group of related proteins with conserved sequence features, i.e. three tandem repeats of about 100 amino acids, each of them with two transmembrane alpha helices connected by a hydrophilic loop and a conserved signature signal sequence motif [33,34]. MCF members are involved in transport of mono-, di- and tricarboxylates, co-factors like NAD^+, FAD and coenzyme A, amino acids, and other substrates necessary for mitochondrial function [35]. *In silico* analysis of the *T. brucei* genome using conserved amino acid sequences and protein domains suggested the presence of 24 MCF proteins [29]. Using previously described and characterized mitochondrial carriers from yeast and humans as a reference, putative functions were assigned to 20 of the predicted MCF proteins in *T. brucei* [29]. However, at present clear biochemical data about their substrate specificities or physiological functions is only available for a few members, one of them being TbMCP5, a *T. brucei* ATP/ADP translocator [36]. Interestingly, a subgroup of MCF proteins shows poor homology to mitochondrial carriers of higher eukaryotes, indicating that they might be unique to kinetoplastids. Bioinformatic analyses using reciprocal BLAST searches revealed that the mitochondrial carrier revealed in our RNAi library screen, TbMCP14, belongs to this subgroup of MCF proteins and is specific to kinetoplastids.

By constructing knock-out mutants, we found that TbMCP14 is not strictly essential for parasite growth in culture. However, growth of *T. brucei* bloodstream forms was clearly reduced in mutants lacking TbMCP14. An even stronger growth phenotype was observed in procyclic form knock-out parasites, showing an almost complete growth arrest after depletion of TbMCP14. Interestingly, we observed that when procyclic form TbMCP14 conditional knock-out parasites were cultured in low glucose medium, growth arrest occurred considerably earlier compared to standard culture conditions. This together with the observed accumulation of the proline metabolite,

pyrroline-5-carboxylate, in parasites over-expressing TbMCP14 suggests that TbMCP14 may be involved in amino acid and energy metabolism in *T. brucei*. It has been described that under glucose-depleted culture conditions, procyclic forms rely primarily on amino acids as substrates for energy production via oxidative phosphorylation [22]. Thus, the observed difference in the time point of growth arrest of parasites between low and normal glucose medium may be due to impaired amino acid metabolism caused by depletion of TbMCP14, resulting in increased dependency of parasites from amino acids. However, TbMCP14 clearly is also necessary when parasites are grown in normal glucose medium, i.e. when most of the ATP is generated by glycolysis. Therefore, the deleterious effect of TbMCP14 cannot be justified by reduced levels of ATP generation; instead, TbMCP14 may be involved in the cross-talk between glycolysis and mitochondrial metabolism. This would explain the later effect on growth when glucose is present.

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Figure Legends

Figure 1. Screening of RNAi library and identification of TbMCP14. A) The concentrations of drugs killing 50% (EC_{50}) and 98% (EC_{98}) of *T. brucei* bloodstream forms were determined using Alamar blue assays. The numbers represent mean values from at least two independent experiments using triplicate determinations. B) Time-line of induction of RNAi library, selection of resistant clones, and sequencing of RNAi inserts from resistant parasites. C) Scheme depicting truncated and full-length TbMCP14. The horizontal grey arrows represent two potential TbMCP14 ORFs, encoding proteins of 344 (truncated) and 401 amino acids (full-length). The colored lines at the bottom indicate the alignments of the RNAi inserts isolated from resistant parasites after selection with G25, T3 and T4 (from top to bottom). The right panel shows an ethidium bromide-stained agarose gel with the RT-PCR products amplified using a primer hybridizing with the spliced leader sequence (SL) together with the gene-specific primers 1 and 2 indicated below full-length TbMCP14.

Figure 2. Sensitivity of *T. brucei* bloodstream forms towards choline analogs after RNAi against TbMCP14. A) Growth curves of RNAi parasites cultured in the absence (squares) or presence (circles) of tetracycline to induce RNAi against TbMCP14. B,C,D) Alamar blue assays to determine the sensitivities of RNAi parasites towards T3 (panel B), G25 (panel C) and T4 (panel D) after down-regulation of TbMCP14 expression. Squares and circles represent trypanosomes cultured in the absence and presence, respectively, of tetracycline (added three days before the assay). The data points represent mean values ± SEM of triplicate determinations from single experiments.

Figure 3. Sensitivity of *T. brucei* procyclic forms towards T3 after modulation of TbMCP14 expression. A) Growth curves of RNAi parasites cultured in the absence (squares) or presence (circles) of tetracycline to induce RNAi against TbMCP14. The inset shows a Northern blot analysis of TbMCP14 mRNA levels from parasites incubated in the absence (-) or presence (+) of tetracycline (upper panels); rRNA levels are shown as loading control (lower panels). B) Alamar blue assays to determine the sensitivity of parasites towards T3 after RNAi-mediated down-regulation of TbMCP14 (panel B), or over-expression of full-length (panel C) or truncated (panel D) TbMCP14. Squares and circles represent trypanosomes cultured in the absence and presence, respectively, of tetracycline. The data points represent mean values ± SEM of triplicate determinations from single experiments. The insets in panels C and D represent SDS-PAGE/immunoblot analyses using anti-cMyc antibody of protein lysates from parasites cultured in the absence (-) or presence (+) of tetracycline to induce over-expression of full-length (panel C, right lane) or truncated (panel D, middle lane) TbMCP14; the right lane in the inset of panel D represents full-length TbMCP14 for size comparison with truncated TbMCP14.

Figure 4. Viability of *T. brucei* bloodstream forms towards G25 after modulation of TbMCP14 expression. Parasites cultured for three days in the absence (left panels) or presence (right panels) of tetracycline to induce over-expression (upper panels) or down-regulation (lower panels) of TbMCP14 were treated with different concentrations of G25 for 24, 48 or 72 h. Digitonin-permeabilized cells were used as a positive control of PI staining (PI positive area of histogram, see materials and methods). The results from one of two separate experiments are shown.

Figure 5. Localization of TbMCP14. *T. brucei* procyclic forms expressing cMyc-tagged TbMCP14 were fixed and stained for DNA with DAPI (first panel), anti-VDAC antibody as mitochondrial marker (second panel), and anti-cMyc to reveal TbMCP14 (third panel). The merge shows mitochondrial co-localization of TbMCP14 and VDAC (fourth panel). Trypanosomes are shown by differential interference contrast (DIC) optics (fifth panel).

Figure 6. Analysis of mitochondrial membrane potential ($\Delta\Psi_m$). *T. brucei* bloodstream forms were incubated in the absence (light grey bars) or presence (dark grey bars) of tetracycline to down-regulate TbMCP14 by RNAi (panel A) or over-express TbMCP14 (panel B) for 3 days before treatment with G25. Parasites were treated with different concentrations of G25 for 24 h followed by addition of 25 nM TMRE for an additional 30 min and analysis by flow cytometry. The values were normalized relative to untreated control trypanosomes. The data points represent mean values ± SD from at least four independent experiments. The asterisks represent statistical difference relative to the respective uninduced control (*$p<0.05$, **$p<0.01$, unpaired student's T-test).

Figure 7. Analysis and growth of *T. brucei* bloodstream and procyclic form TbMCP14 null mutants. A) In bloodstream form (bs) parasites, the two endogenous TbMCP14 alleles (∞, 1363 bp) were replaced by hygromycin (*, 2762 bp,) and blasticidin (#, 2135 bp) resistance genes, whereas in procyclic form (pc), the endogenous alleles were replaced by blasticidin (#, 2135 bp) and phleomycin (#, 2111 bp) resistance genes. B) Growth of *T. brucei* bloodstream form TbMCP14 null mutants in culture (circles), in comparison with the parental cell line (squares). C) Growth of *T. brucei* procyclic form TbMCP14 conditional null mutants in the presence (squares) or absence (circles) of tetracycline. D) SDS-PAGE/immunoblot analysis of lysates from *T. brucei* procyclic form TbMCP14 conditional null mutants grown in the presence (+) or absence (-) of tetracycline for indicated times. cMyc-tagged TbMCP14 was visualized with anti-cMyc antibody. Hsp70 was visualized with anti-HSP70 antibody and represents a loading control. Molecular mass markers are indicated in the margin.

Figure 8. Effect of depletion of TbMCP14 on growth of *T. brucei* procyclic forms cultured in glucose-depleted medium. A) Parasites were grown in SDM80 containing normal glucose (5.5 mM; dashed lines) or low glucose (0.15 mM; solid lines) in the absence (black symbols) or presence (red symbols) of tetracycline to induce RNAi-mediated down-regulation of TbMCP14. B,C) TbMCP14 conditional

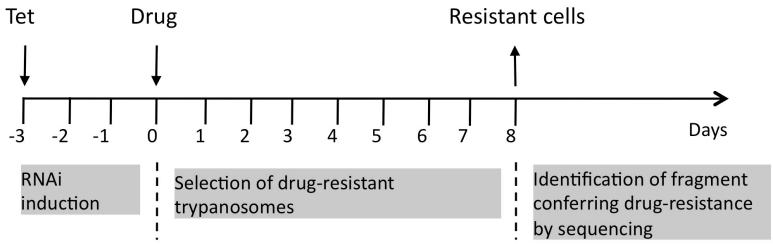
knock-out parasites were grown in glucose-depleted (B) or glucose-containing medium (C) in the presence (black symbols) or absence (red symbols) of tetracycline to maintain or deplete, respectively, TbMCP14.

Figure 1

A

	G25	T3	T4
EC_{50} (µM)	0.06	1.22	0.09
EC_{98} (µM)	0.160	3.5	0.5

B



C

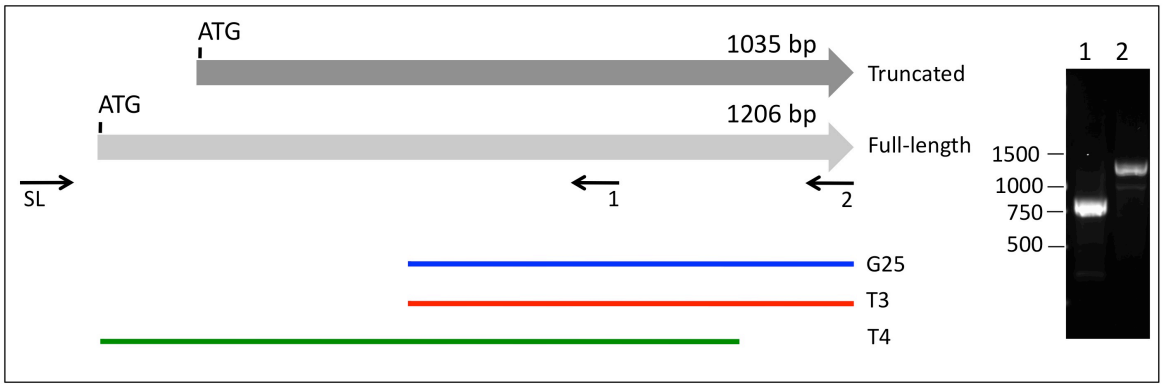


Figure 2

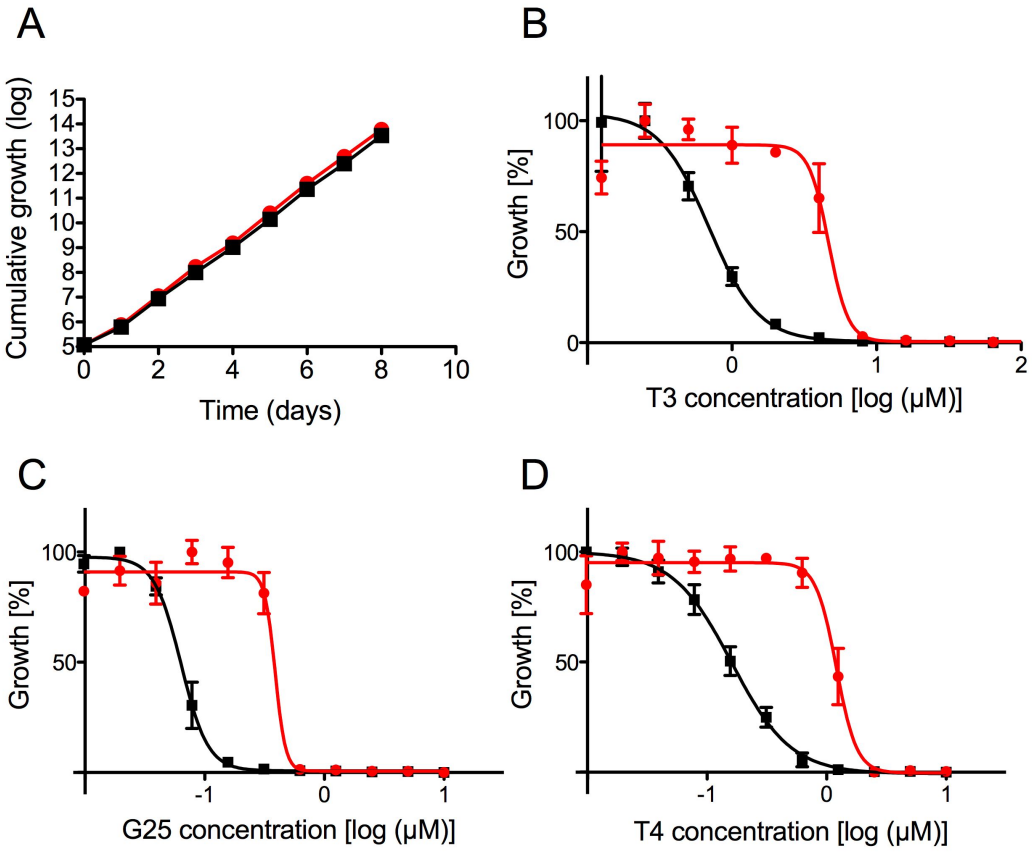

A
Cumulative growth (log)
15
14
13
12
11
10
9
8
7
6
5
0
2
4
6
8
10
Time (days)
B
Growth [%]
100
50
0
0
1
2
T3 concentration [log (µM)]
C
Growth [%]
100
50
-1
0
1
G25 concentration [log (µM)]
D
Growth [%]
100
50
-1
0
1
T4 concentration [log (µM)]

Figure 3

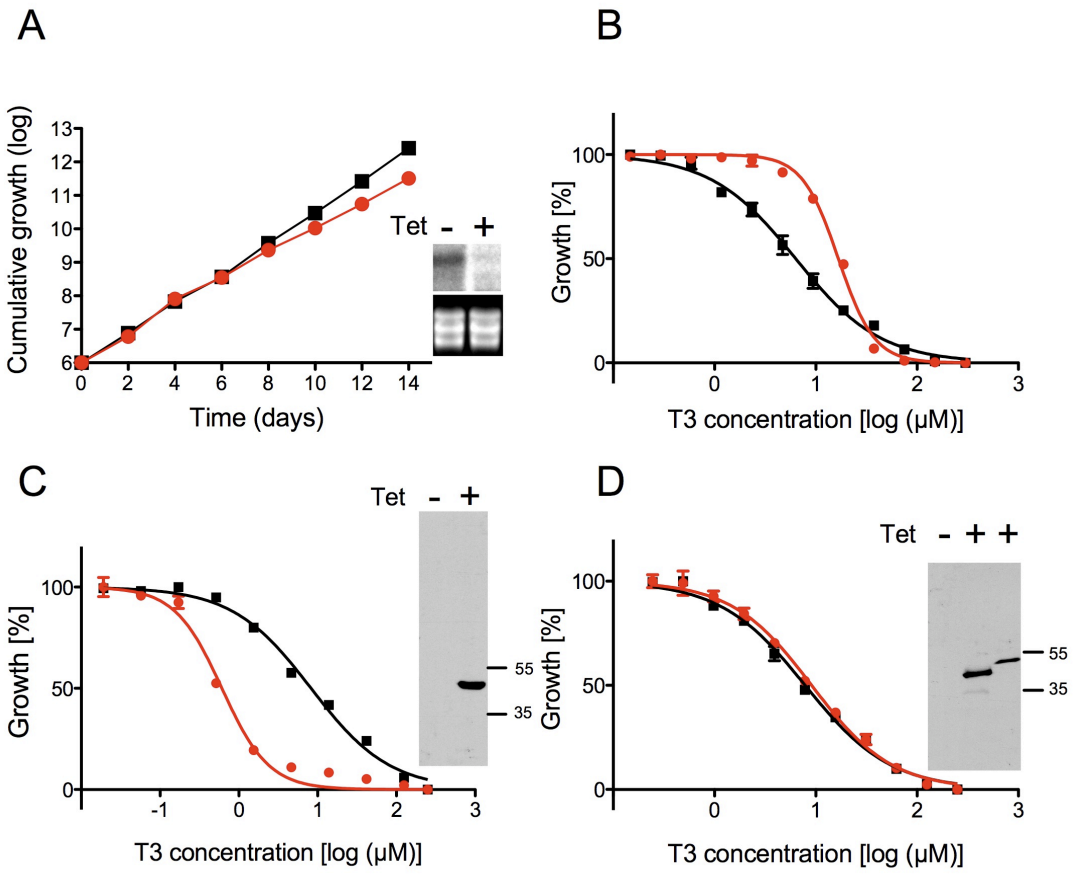

A
Cumulative growth (log)
Time (days)
Tet - +
B
Growth [%]
T3 concentration [log (μM)]
C
Growth [%]
T3 concentration [log (μM)]
Tet - +
55
35
D
Growth [%]
T3 concentration [log (μM)]
Tet - + +
55
35

Figure 4

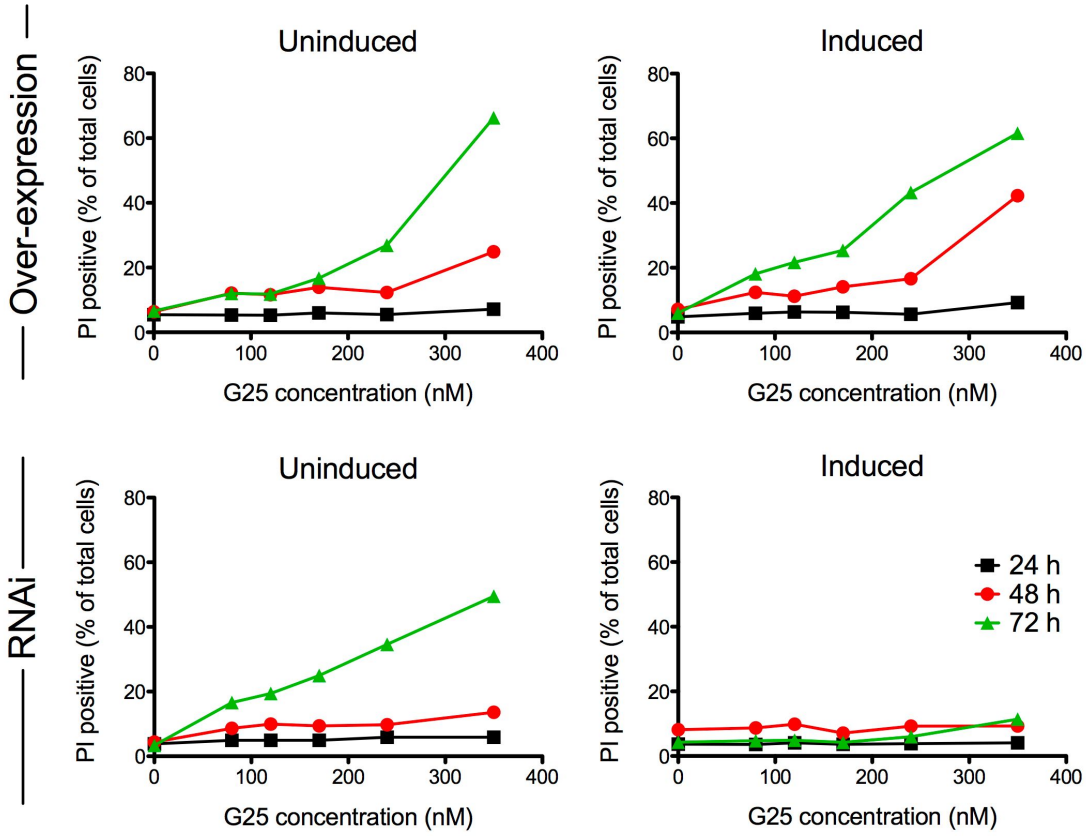

Over-expression
RNAi
Uninduced
Induced
PI positive (% of total cells)
G25 concentration (nM)
0
20
40
60
80
100
200
300
400
24 h
48 h
72 h

Figure 5

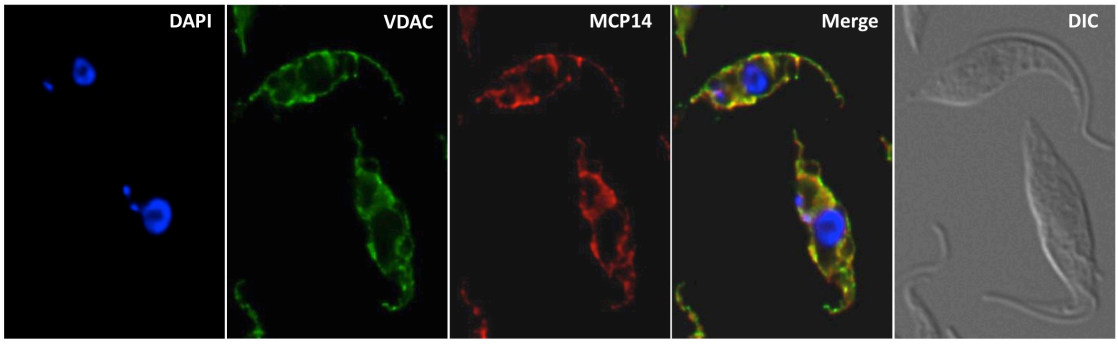


Figure 6

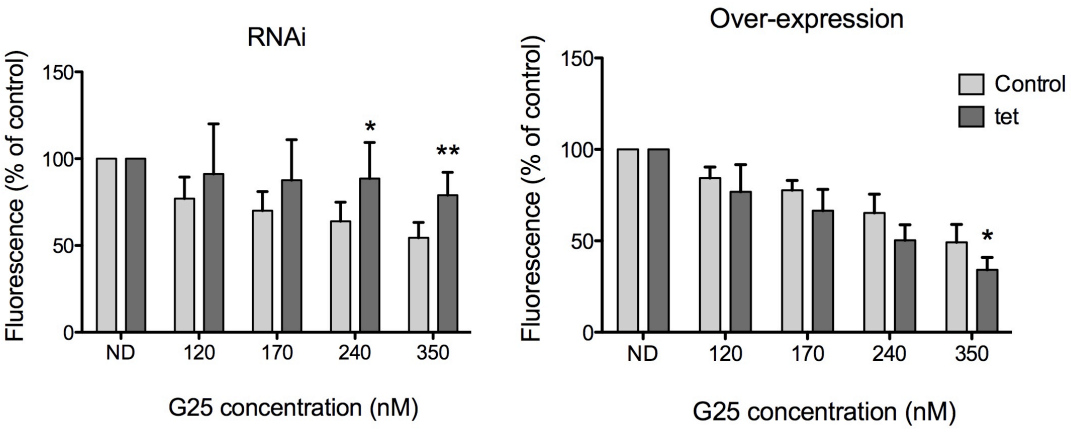

RNAi
Fluorescence (% of control)
150
100
50
0
ND
120
170
240
350
*
**
G25 concentration (nM)
Over-expression
Control
tet
Fluorescence (% of control)
150
100
50
0
ND
120
170
240
350
*
G25 concentration (nM)

Figure 7

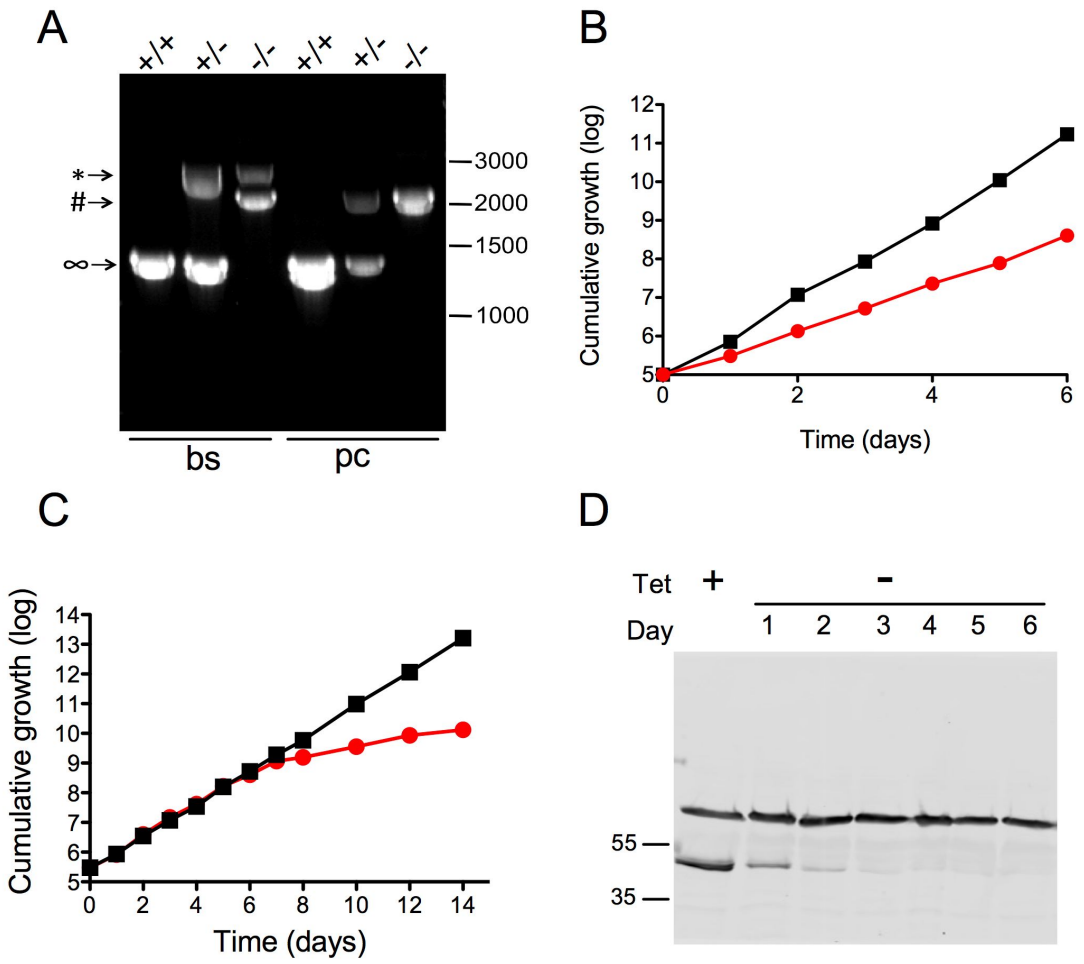
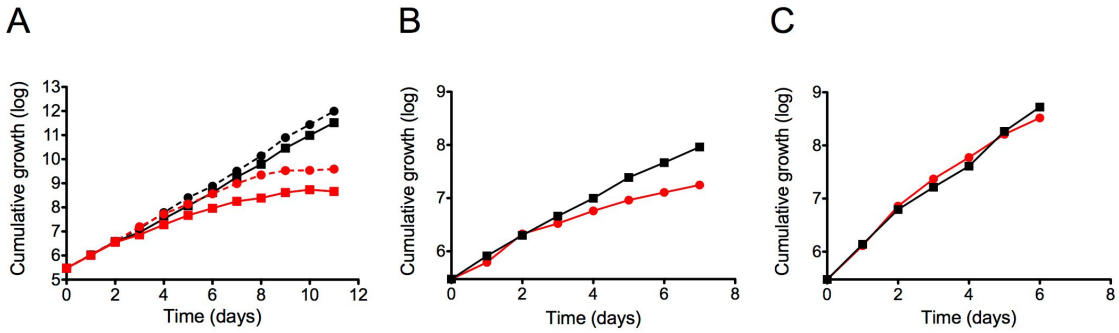

A
+/+
+/-
-/-
+/+
+/-
-/-
*→
#→
∞→
3000
2000
1500
1000
bs
pc
B
Cumulative growth (log)
Time (days)
C
Cumulative growth (log)
Time (days)
D
Tet
+
-
Day
1 2 3 4 5 6
55
35

Figure 8

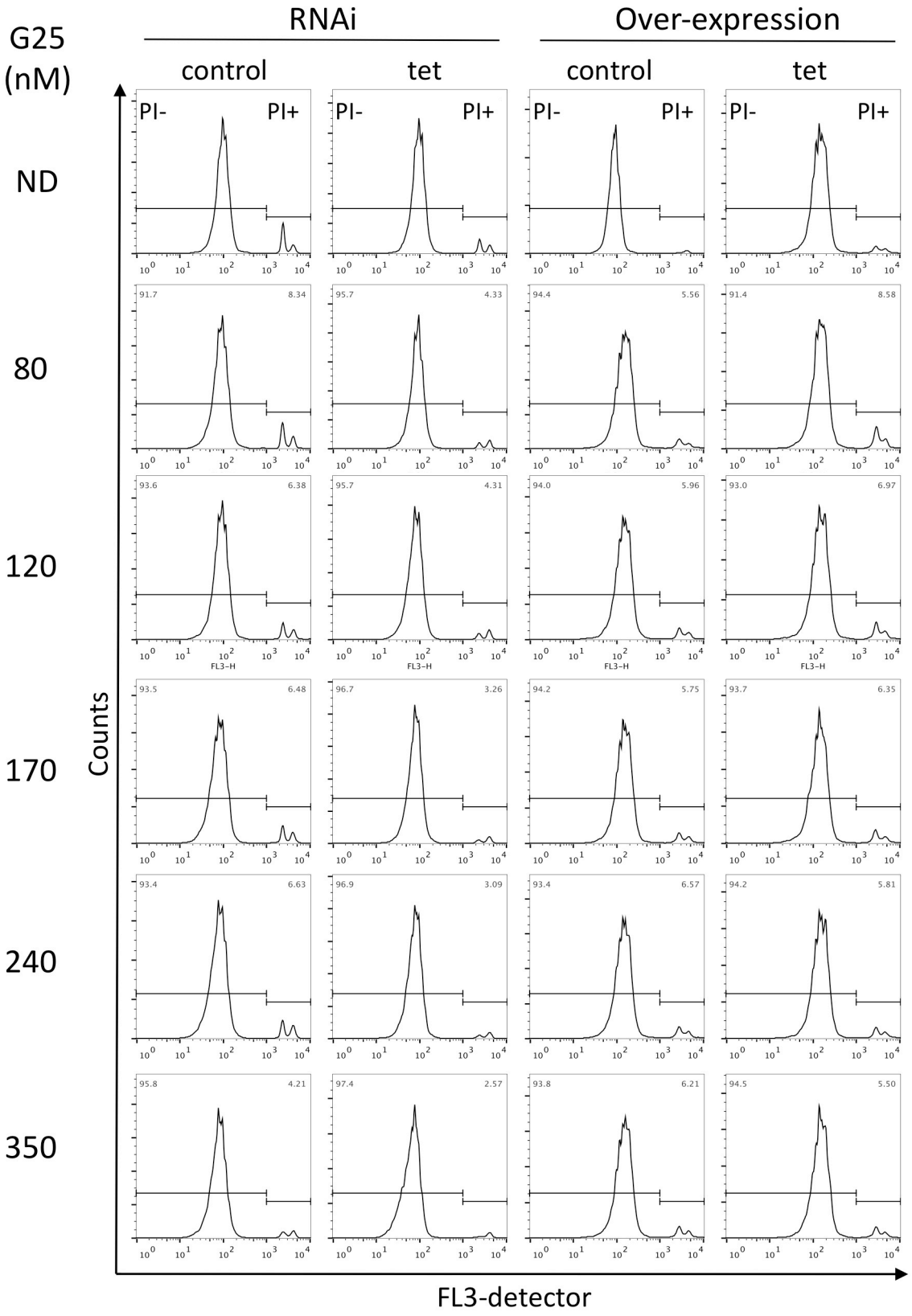

A
B
C
Cumulative growth (log)
Time (days)
0
2
4
6
8
10
12
5
6
7
8
9
10
11
12
13

Supplementary Figures

Table S1 – Oligonucleotides used.

Oligonucleotid name	Sequence
Primer 1 TbMCP14-F	5'- GCCCAAGCTTGGATCCGAGGGGTCTTCGGGCTATTTA -3'
Primer 2 TbMCP14-R	5'- TGGCTCTAGACTCGAGCAGAAGCCCAGCGTAGAGAC -3'
Primer 3 TbJPM14O-F (full-length)	5'- ACGCAAGCTTATGAGTATCACCAACATCGCTG -3'
Primer 4 TbJPM14O-R	5'- CGCGGATCCTATTTGTTTCCAGAGTTCTCGGTC -3'
Primer 5 TbJPM14O-F (truncated)	5'- ACGCAAGCTTATGCTACGAACGTTGCTGCTG-3'
Primer 6 MCP14KO-5'_fw	5'- CCCCTCGAGAACGGATTTATGGAGTTTATTTTTC -3'
Primer 7 MCP14KO-5'_rv	5'- GCCCAAGCTTCTAACTTAATGTGAGGACGTC -3'
Primer 8 MCP14KO-3'_fw	5'- TGGCTCTAGAAGGAGAAATGGTGTGGTACAC -3'
Primer 9 MCP14KO-3'_rv	5'- CCCGCGGCCGCTGGTGATTAAACCTCACTTTATC -3'
Primer 10 MCP14qPCR_fw	5'- CGAAACCTCCATCCGTATTC-3'
Primer 11 MCP14qPCR_rv	5'- CCACGATAAATAGCCCGAAG-3'
Primer 12 SpliceLeader	5'- CGCTATTATTAGAACAGTTTCTGTAC -3'

Supplementary Figure 1. Viability of parasites used in mitochondrial membrane potential measurements (Figure 4). *T. brucei* bloodstream forms towards G25 treatment for 24 h and after modulation of TbMCP14 expression. Parasites cultured for three days in the absence (control) or presence (tet) of tetracycline to induce down-regulation (left panels) or over-expression (right panels) of TbMCP14 were treated with different concentrations of G25 for 24 h. Percentages of propidium iodide impermeable (PI-) or permeable (PI+) parasites are shown. Digitonin-permeabilized cells were used as a positive control of PI staining (PI+ area of histogram, see materials and methods). The results from one of two separate experiments are shown.


G25 (nM)
RNAi
Over-expression
control
tet
control
tet
PI-
PI+
ND
80
120
170
240
350
91.7
8.34
95.7
4.33
94.4
5.56
91.4
8.58
93.6
6.38
95.7
4.31
94.0
5.96
93.0
6.97
93.5
6.48
96.7
3.26
94.2
5.75
93.7
6.35
93.4
6.63
96.9
3.09
93.4
6.57
94.2
5.81
95.8
4.21
97.4
2.57
93.8
6.21
94.5
5.50
FL3-H
Counts
FL3-detector

3. Additional results

3.1 Effective concentration of G25 doesn't inhibit PC synthesis

Our results (summarized in Chapters 2.1 and 2.2) strongly suggest that the main target of choline analogs in *T. brucei* is not lipid biosynthesis but mitochondria. This was also confirmed when I assessed biosynthesis of PC by *T. brucei* bloodstream forms in presence of effective concentration of G25 (0.16 µM). To do that, parasites were pre-incubated or not with 0.16 or 10 µM G25 during 21 h. After three hours incubation in presence of [^{3}H]choline (2 µCi/ml) and [^{3}H]ethanolamine (0.25 µCi/ml, as control), total lipids were extracted and cell equivalent amounts were applied in a TLC plate (as described in [85]).

This lipids analysis showed that at very high concentration (10 µM), G25 specifically inhibits PC biosynthesis by *T. brucei* bloodstream forms (Figure 15, middle panel), supporting our previous results ([85] manuscript in Chapter 2.2). Nevertheless, treatment with effective concentration of G25 (0.16 µM, EC_{98} found in Chapter 2.2) during 24 h doesn't affect PC biosynthesis (Figure 15, lower panel). The integral of resulting peaks after TLC analysis corresponding to PC and PE were calculated and the ratio PE/PC has not changed between samples in absence or in presence of 0.16 µM G25 (Figure 15). These results clearly demonstrate that the toxic effect of G25 is not related to inhibition of uptake of [^{3}H]choline and/or PC biosynthesis.

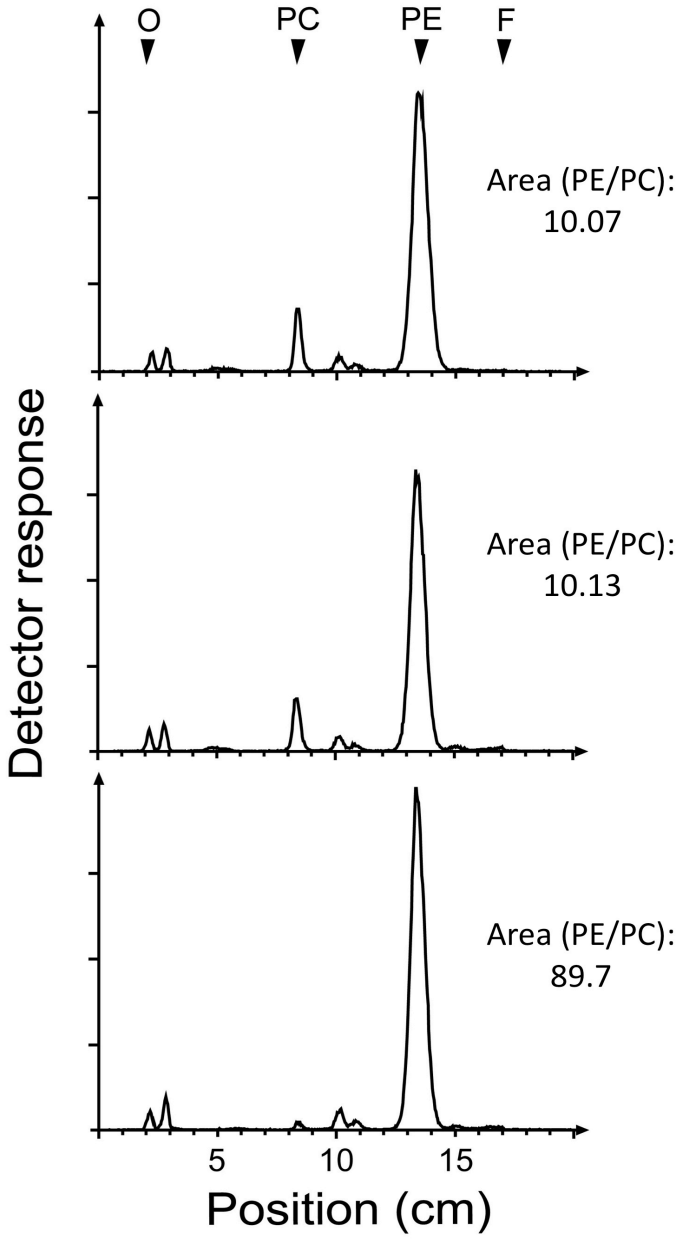


Figure 15. Effect of G25 on biosynthesis of [^{3}H]phosphatidylcholine (PC).*T. brucei* bloodstream forms (2 x 10^7 parasites in 10 ml culture medium) in absence (top panel) or in presence of 0.16 µM (middle panel) and 10 µM (bottom panel) for 21 h were subsequently incubated for extra 3 h in presence of 2 μCi/ml [^{3}H]choline and 0.25 μCi/ml [^{3}H]ethanolamine (as control). Afterwards, lipids were extracted and analyzed by TLC, and radiolabeled products were detected by radioisotope scanning. The area under peaks PE and PC were calculated and the ratio PE/PC is shown for each case. The arrows indicate the migration of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) standards run on the same TLC plate; O, site of sample application; F, migration distance of solvent front.

3.2 Effect of G25 on dyskinetoplastic trypanosomes

Despite the existence of natural dyskinetoplastic trypanosomes and the induced loss of kDNA in laboratory conditions, the mitochondrial DNA in *T. brucei* is essential and mechanisms involved in its replication are considered valid drug targets [138]. In bloodstream forms, the F_1F_o-ATP synthase is working in the reverse direction hydrolyzing ATP to produce the proton gradient between the mitochondrial matrix and the cytosol [60,62,63]. The subunit A6 of the membrane-embedded F_0 component is encoded by the kDNA [58]. Since choline analogs affect mitochondrial membrane potential, we hypothesized whether this could be a result of alterations in kDNA. Therefore, we used bloodstream forms that tolerate dyskinetoplasy (L262P mutant [59]; kindly provided by A. Schnauffer) to test G25 sensitivity. These cells bear a mutation in the nuclear-DNA-encoded γ subunit of the F_1 component of the F_1F_o-ATP synthase that compensates for loss of kDNA [59]. This mutation in the γ subunit allows the generation of $\Delta\Psi_m$ by the 'truncated' A6-defective F_1F_o-ATP synthase.

To test our hypothesis, I used L262P mutant bloodstream forms before and after treatment with acriflavine or ethidium bromide, DNA-intercalating compounds that induce loss of kDNA [138,139]. Our findings indicated that L262P mutant bloodstream forms are still sensitive to G25 with comparable EC_{50} to control parasites (Figure 16). Moreover, sensitivity is not changed when loss of kDNA is induced before incubation with G25, but the hill coefficient of acriflavin- or ethidium bromide-treated L626P mutants is changed, probably due to secondary effects caused by pre-incubation with acriflavin or ethidium bromide. However, since there is no shift in resistance to G25, kDNA is unlikely to be the main target of this choline analog.

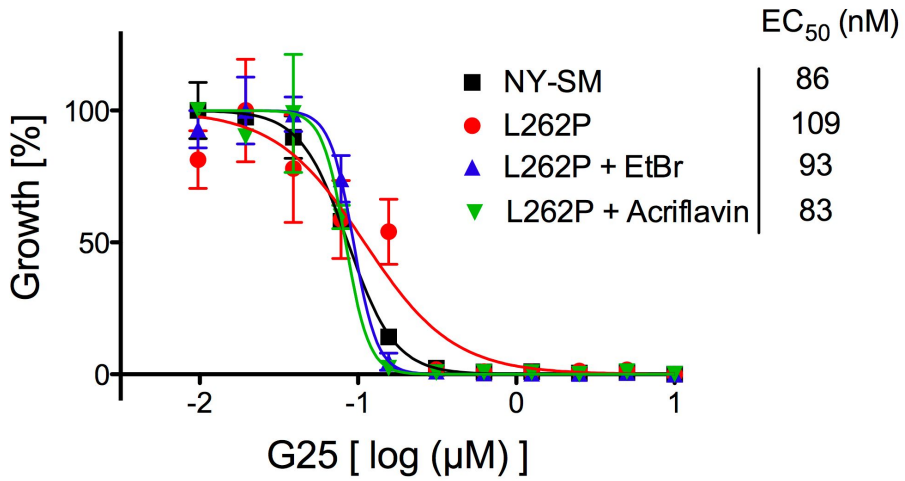


Figure 16. Sensitivity of dyskinetoplastic bloodstream forms to G25. Dyskinetoplasy-tolerant bloodstream forms (L262P) were pre-treated or not with 20 nM acriflavin or 10 nM ethidium bromide (EtBr) until kinetoplast could no longer be detected by fluorescence microscopy using DAPI staining. NY-SM, control bloodstream forms (containing the kinetoplast). Data points are mean values ± SEM of three determinations.

3.3 T1 exerts a different mode of action

Apart from M53, all choline analogs depicted in Figure 13 were tested against *T. brucei in vitro* after down-regulation of TbMCP14. This group of choline analogs was shown to have similar mode of action, except T1 that showed a divergent mechanism. In contrast to G25, a bis-quaternary ammonium, and T3, a bis-thiazolium salt, T1 is an asymmetric monocationic molecule, which contains only one thiazolium ring (see Figure 13 for structures). Drug-sensitivity assays revealed an EC_{50} of 1.35 ± 0.08 µM for T1 (results from two independent experiments), and interestingly, down-regulation of TbMCP14 did not confer resistance to T1 (Figure 17A) indicating that it may exert toxicity by a different mechanism. Based on that, we investigated weather T1 could affect $\Delta\Psi_m$. We assessed $\Delta\Psi_m$ of bloodstream forms using TMRE (Figure 17B) and found that, in contrast to G25, 8 h of treatment with 0.15-5 µM of T1 had no affect on $\Delta\Psi_m$. Furthermore, at 10 µM, T1 decreased the $\Delta\Psi_m$ to basal levels only after 30 min of treatment (not shown).

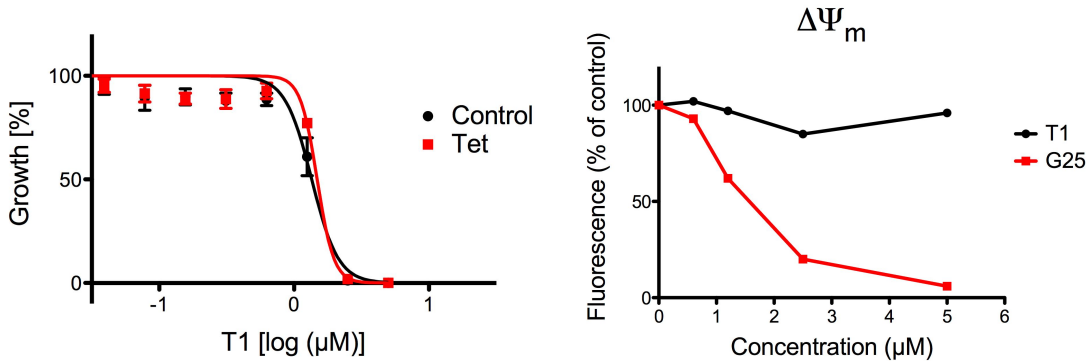


Figure 17. Sensitivity of trypanosomes to T1 after down-regulation of TbMCP14 (A) and effect on $\Delta\Psi_m$ (B). A)TbMCP14 RNAi cells were induced (Tet) three days before adding different concentrations of T1 (for more details see Materials and Methods section of Manuscript B). The results of one from two independent experiments are shown. Data points reflect mean ± SEM from three determinations. B) Bloodstream form (NY-SM) parasites were incubated in presence of T1 or G25 for 8 h before addition of TMRE and analysis of $\Delta\Psi_m$ by flow cytometry. Experimental details can be found in Materials and Methods, Chapter 2.2.

A second piece of evidence that T1 may have a different mechanism of action came from drug sensitivity assays using yeast. As mentioned in chapter 1.10, there is experimental evidence that T16 and T3 are taken up Plasmodium via a choline transporter [83,103]. Thus, we assessed toxicity of selected choline analogs using choline transporter-deficient yeast (hnm1) [140]. Our results showed that the hnm1 knock-out mutant is more resistant to T1 (Figure 18A) indicating that T1 could indeed be transported by hnm1 in yeast. In contrast, drug sensitivity assays in *T. brucei* in presence of

excessive amounts of choline or HC-3, a specific inhibitor of choline uptake, has no effect on T1 toxicity. Therefore, T1 may not use a choline carrier to cross the plasma membrane of trypanosomes. In contrast, hnm1 mutant yeast are more susceptible to T3 and T4 compared to hnm1-complemented control cells. These results strongly suggest distinct modes of action of T1 in *T. brucei* and yeast. However, is still unclear why hnm1-complemented yeast is more resistant to T3 and T4.

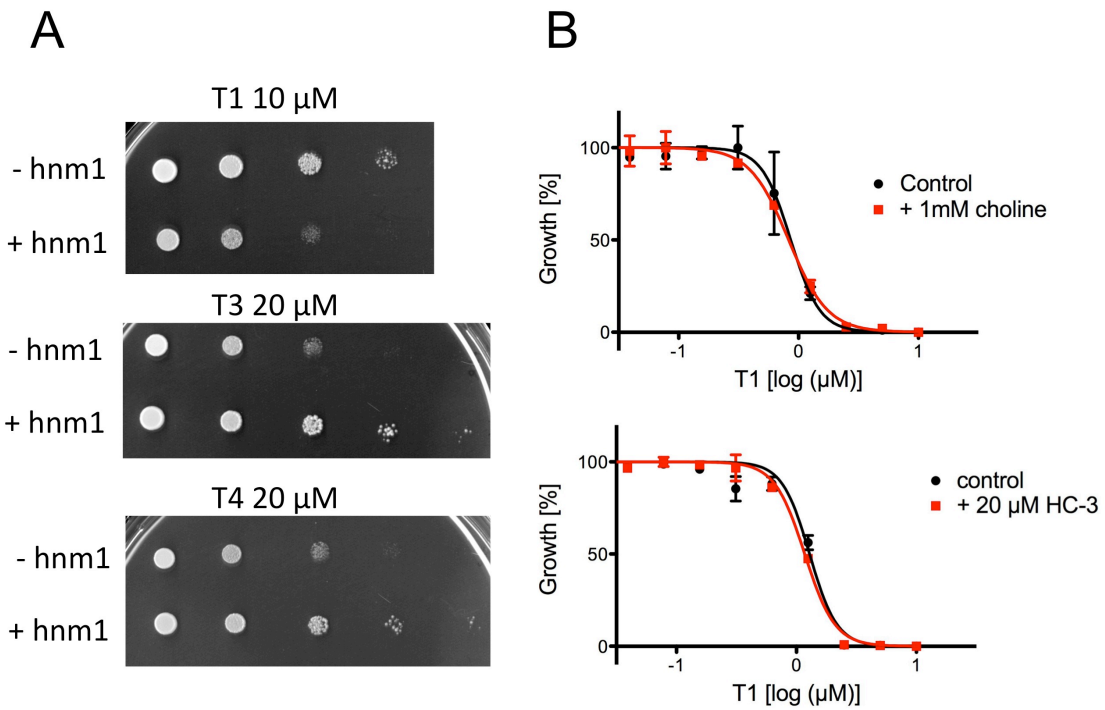


Figure 18. Sensitivity of choline transporter-deficient (- hnm1) and -complemented (+ hnm1) yeast (A) and *T. brucei* bloodstream forms (B) to choline analogs. A) Growth in plates containing the indicated concentrations of T1, T3 or T4 for three days after serial dilutions (1:10, experiment was performed in collaboration with C. Wirdnam, laboratory of D. Rentsch). B) Drug sensitivity of bloodstream forms to T1 in presence of excessive amounts of choline (+ 1 mM choline) or hemicholinium-3 (+ 20 µM HC-3). The results of one from two independent experiments are shown. Data points reflect mean ± SEM from three determinations.

3.4 TbMCP14 and mode of action of pentamidine

The mode of action of diamidines is possibly multi-factorial, but there is evidence that pentamidine and other fluorescent diamidines may accumulate inside mitochondria and cause a decrease of $\Delta\Psi_m$ of kinetoplastids [141,142,143,144]. For this reason, we studied if TbMCP14 may be involved in the mode of action of diamidines. We assessed the sensitivity of trypanosomes to two

diamidines, pentamidine and DB829 (Figure 19A), after down-regulation or over-expression of TbMCP14. The results show no significant shift in parasite sensitivity towards pentamidine (Figure 19B) and DB829 (not shown) after depletion of TbMCP14. Since quantitative PCR showed a 70% down-regulation of TbMCP14 mRNA, we cannot exclude the possibility that residual expression of TbMCP14 contributes to toxicity of pentamidine and DB829. Interestingly, over-expression of TbMCP14 in procyclic forms resulted in a 3-fold more susceptible phenotype towards pentamidine (Figure 19C). These results were comparable to the effect of TbMCP14 over-expression on choline analogs toxicity (Chapter 2.2, Figure 3C). Together, these preliminary findings suggest that TbMCP14 may be involved in the mode of action of diamidines.

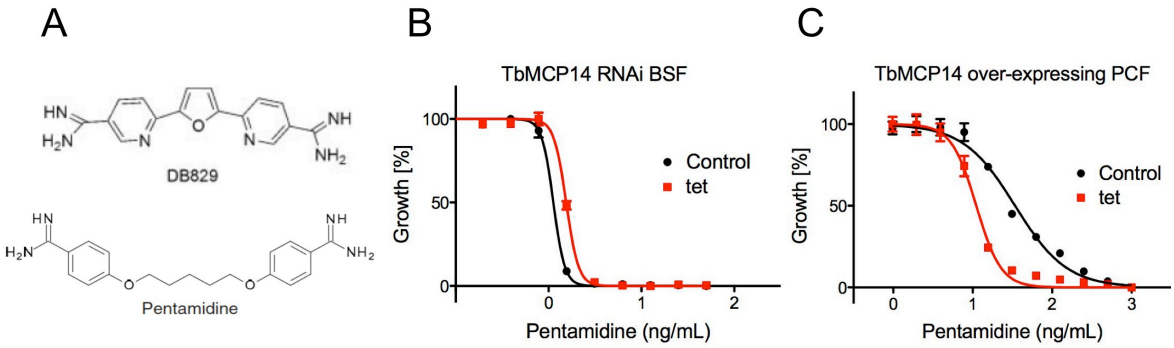


Figure 19. Structure of diamidines and sensitivity of trypanosomes to pentamidine. A) Chemical structures of DB829 and pentamidine. B) Sensitivity of bloodstream forms to pentamidine after inducing down-regulation of TbMCP14 (tet) or not (control). C) Sensitivity of procyclic forms to pentamidine after inducing over-expression of TbMCP14 (tet) or not (control). B and C display results of one from two independent experiments. Data points show mean values ± SEM from three determinations.

3.5 Insights from metabolomics analyses on G25 effect

In order to get clues about the mode of action of G25 we decided to run an untargeted metabolomics analysis, in collaboration with Michael Barrett (University of Glasgow), of bloodstream forms treated or not with the EC_{98} concentration of G25 (160 nM). Parasites were cultured in presence of G25 for 48 h (done in triplicates), an incubation time that doesn't affect growth, and total metabolites from 10^8 parasites were subsequently extracted with chloroform:methanol:water (1:3:1) and analyzed by LC-MS. This method revealed global changes in metabolism of bloodstream forms after treatment with G25. An unbiased analysis indicated changes in amino acid levels, peptides and lipid metabolism (Figure 20A). In addition, to detect further alterations in specific metabolites, the data was sorted according to significance (e.g. $p < 0.05$), resulting in the identification of two intermediates of the tri-carboxylic acid cycle, malate and 2-oxoglutarate, which are inversely changed in G25-treated parasites. The relative amount of malate was reduced to 0.46

whereas that of 2-oxoglutarate was increased by 3.16, which could be explained by the action of a putative malate/2-oxoglutarate carrier in trypanosomes [135]. Another changed metabolite in carbohydrate metabolism is deoxyribose, which was found to be 1.87X increased in G25-treated parasites (Figure 21). Several metabolites involved in amino acid metabolism were significantly changed after G25 treatment, especially the ones related to metabolism of glutamate, proline, aspartate and arginine. In lipid metabolism, only 3 metabolites were significantly changed, but without apparent correlation between them. In contrast, accumulation of several metabolites from nucleotide metabolism was observed (Figure 21). In summary, our metabolomics analysis of bloodstream form parasites treated with G25 indicated changes in amino acids metabolism, in addition to scattered changes in peptide and carbohydrate metabolisms, and small changes in metabolites involved in nucleotide and lipid metabolism. However, the relative high noise makes it difficult to conclude whether the changes are real or not. The accuracy of the analysis could be improved by performing time-course experiments in the presence of G25, but metabolomics analyses are costly and time-consuming.

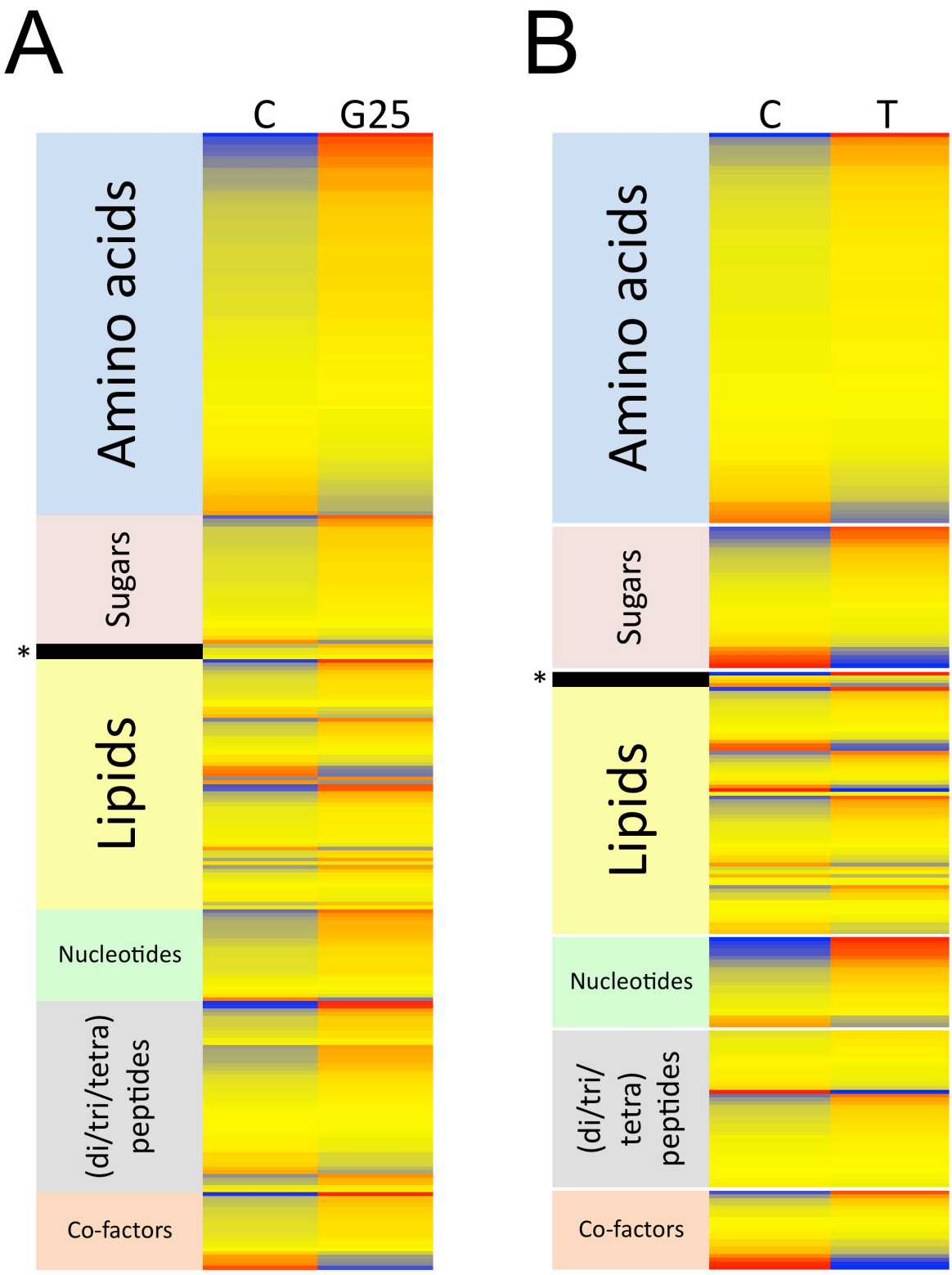


Figure 20. Heat maps of changes in abundance of putative metabolites relative to untreated control cells (C). A) Metabolomics analysis of bloodstream forms treated (G25) or not (C) with 160 nM G25 for 48 h. B) Metabolomics analysis of procyclic forms after over-expression of TbMCP14 (T) compared to control (C). *Red*, increased; *blue*, decreased; *yellow*, unchanged. *putative metabolites involved in oxidative phosphorylation.

N-Succinyl-L-glutamate	8	Amino Acid Metabolism	1.00	**3.47**
N-Acetyl-L-aspartate	8	Amino Acid Metabolism	1.00	**2.94**
L-Glutamate	10	Amino Acid Metabolism	1.00	**2.32**
(R)-2-Hydroxyglutarate	10	Amino Acid Metabolism	1.00	**2.30**
(S)-2-Aceto-2-hydroxybutanoate	8	Amino Acid Metabolism	1.00	**1.85**
S-glutathionyl-L-cysteine	10	Amino Acid Metabolism	1.00	**1.75**
(S)-Methylmalonate semialdehyde	8	Amino Acid Metabolism	1.00	**1.56**
Choline	10	Amino Acid Metabolism	1.00	**1.36**
2-Hydroxy-3-(4-hydroxyphenyl)propenoat	8	Amino Acid Metabolism	1.00	**1.16**
L-Glutamate 5-semialdehyde	8	Amino Acid Metabolism	1.00	**0.89**
L-Arginine phosphate	8	Amino Acid Metabolism	1.00	**0.52**
2-Oxoglutarate	10	Carbohydrate Metabolism	1.00	**3.16**
Deoxyribose	8	Carbohydrate Metabolism	1.00	**1.87**
[FA trihydroxy(4:0)] 2,3,4-trihydroxy-butanoic acid	8	Carbohydrate Metabolism	1.00	**1.38**
alpha-D-Glucosamine 1-phosphate	8	Carbohydrate Metabolism	1.00	**1.29**
Pyruvate	10	Carbohydrate Metabolism	1.00	**1.18**
(S)-Malate	10	Carbohydrate Metabolism	1.00	**0.46**
Orthophosphate	10	Energy Metabolism	1.00	**1.60**
Taurine	10	Lipid Metabolism	1.00	**0.63**
[PR] (+)-15-nor-4-thujopsen-3-one	7	Lipids: Prenols	1.00	**1.84**
SM(d18:1/14:0)	7	Lipids: Sphingolipids	1.00	**1.41**

(S)-6-Hydroxynicotine	7	Metabolism of Cofactors and	1.00	**1.50**
Isopyridoxal	8	Metabolism of Cofactors and	1.00	**1.19**
3'-AMP	8	Nucleotide Metabolism	1.00	**1.85**
Adenine	10	Nucleotide Metabolism	1.00	**1.68**
dTDP	8	Nucleotide Metabolism	1.00	**1.68**
Pseudouridine 5'-phosphate	8	Nucleotide Metabolism	1.00	**1.64**
Guanosine 3'-phosphate	8	Nucleotide Metabolism	1.00	**1.59**
Guanine	10	Nucleotide Metabolism	1.00	**1.43**
Cytidine	10	Nucleotide Metabolism	1.00	**1.24**
Pseudouridine	8	Nucleotide Metabolism	1.00	**1.19**
Asp-Arg	7	Peptide(di-)	1.00	**5.32**
Glu-Arg	7	Peptide(di-)	1.00	**4.66**
Lys-Lys	7	Peptide(di-)	1.00	**1.96**
Leu-Lys-Gly	7	Peptide(tri-)	1.00	**2.18**
Leu-Met-Gly	7	Peptide(tri-)	1.00	**1.70**

Figure 21. List of significantly ($p < 0.05$) changed metabolites in *T. brucei* bloodstream forms after G25 treatment (160 nM) for 48 h.

3.6 Metabolomics analysis after over-expression of TbMCP14

Additionally, we also performed an untargeted metabolomics analysis using *T. brucei* procyclic forms after down-regulation or over-expression of TbMCP14. Unfortunately, the analysis performed with TbMCP14 RNAi parasites produced inconclusive results, possibly because of the experimental conditions; i.e. in SDM79 with three days after induction, under these conditions there is a late and mild growth defect after down-regulation of TbMCP14 (see Chapter 2.2, Figure 3).

An unbiased analysis of changed metabolites in procyclic forms over-expressing TbMCP14 indicated more changes, in metabolism of carbohydrates, nucleotides and several co-factors (Figure 20B). Interestingly, after filtering the data according to significance ($p < 0.05$), a significant increase of 4 times of 1-pyrroline-5-carboxylate (1P5C), a metabolite related to the proline degradation pathway, was detected (Figure 22), suggesting an involvement of TbMCP14 in proline and glutamate metabolism (Figure 23 and discussion section of Chapter 2.2). The table displayed in Figure 22 shows all significantly ($p < 0.05$) changed metabolites after over-expression of TbMCP14. Minor changes in lipids and nucleotides metabolism were also detected but without a clear correlation with previous analyses.

(S)-1-Pyrroline-5-carboxylate	8	Amino Acid Metabolism	1.00	**3.99**
L-Aspartate	10	Amino Acid Metabolism	1.00	**1.84**
(2S)-2-Isopropylmalate	8	Amino Acid Metabolism	1.00	**1.47**
2-Phenylacetamide	8	Amino Acid Metabolism	1.00	**1.22**
S-Adenosyl-L-methionine	8	Amino Acid Metabolism	1.00	**1.19**
L-Threonine	10	Amino Acid Metabolism	1.00	**1.09**
L-Alanine	10	Amino Acid Metabolism	1.00	**1.08**
Succinate	10	Carbohydrate Metabolism	1.00	**1.15**
Docosahexaenoicacid	7	Lipids: Fatty Acyls	1.00	**1.35**
2-Butenoate	7	Lipids: Fatty Acyls	1.00	**1.13**
6-Hydroxypseudooxynicotine	7	Metabolism of Cofactors and Vitamins	1.00	**1.93**
2-(Methylthio)ethanesulfonate	7	Metabolism of Cofactors and Vitamins	1.00	**1.46**
CDP	8	Nucleotide Metabolism	1.00	**1.79**
dTDP	8	Nucleotide Metabolism	1.00	**1.57**
Orotate(Fragment)	8	Nucleotide Metabolism	1.00	**1.20**
Ile-Lys-Lys-Ser	7	Peptide(tetra-)	1.00	**1.57**

Figure 22. List of significantly ($p < 0.05$) changed metabolites after over-expression of TbMCP14 in *T. brucei* procyclic forms.

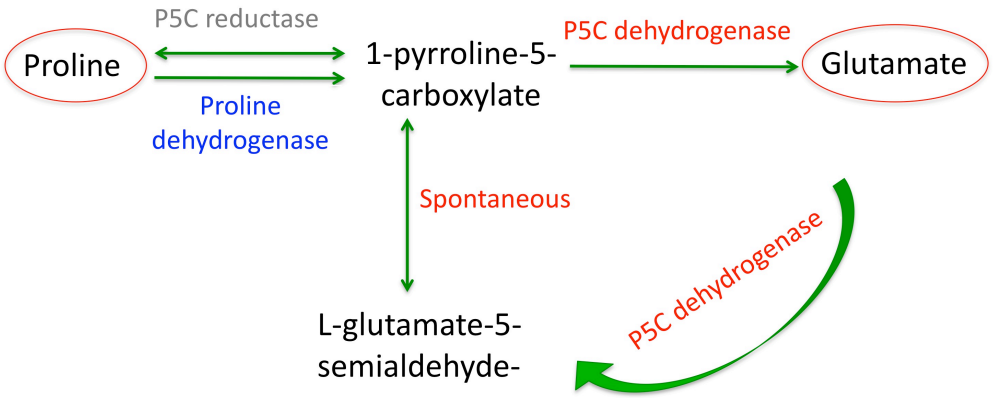


Figure 23. Initial steps of proline metabolism in mitochondria (based on KEGG pathway database).

Considering these results, we reasoned that TbMCP14 may function as a proline carrier. Hence, in collaboration with Moritz Niemann and André Schneider (University of Bern), I prepared pure extracts of mitochondria of TbMCP14 conditional knock-out after 5 days in presence or absence of tetracycline. Isolated mitochondria were used to perform uptake assays using radiolabeled proline or the choline analog T3 (not shown). Unfortunately, this approach produced inconclusive results.

3.7 TbMCP14 and proline metabolism

Three observations encouraged us to investigate a possible involvement of TbMCP14 in metabolism of proline. First, G25 has a nitrogen-containing ring very closely related to proline (Figure 24A). Second, metabolomics analysis done with procyclic forms over-expressing TbMCP14 showed an accumulation of 1-pyrroline-5-carboxilate (1P5C), a metabolite of proline. Finally, experiments performed in glucose-depleted medium (Chapter 2.2) suggested that TbMCP14 may be involved in energy metabolism. Therefore, RNAi against TbMCP14 was induced and uptake of radiolabeled [U-^{14}C]proline into trypanosomes was determined. We found that down-regulation of TbMCP14 promoted an increased incorporation of proline when parasites were cultivated in standard medium (SDM79, Figure 24B) or glucose-depleted medium (not shown).

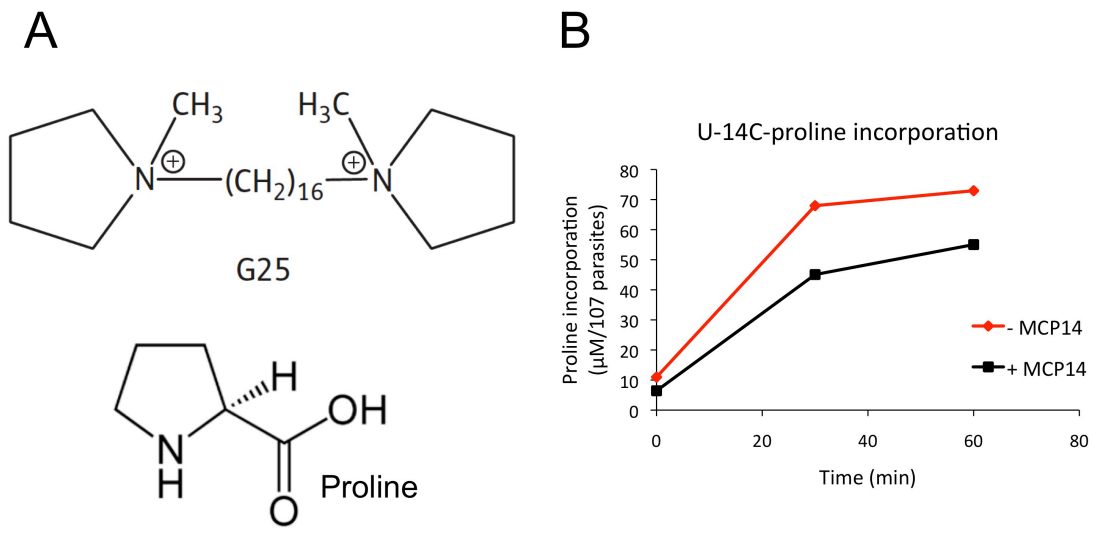


Figure 24. A) Chemical structures of G25 and proline. B) [U-^{14}C]proline incorporation by TbMCP14 KO procyclic forms. Parasites were cultivated in standard medium (6 mM glucose) with (black squares) or without (red diamonds) tetracycline for 5 days, collected and incubated in PBS (+5 mM glucose) containing 400 µM non-labeled and 2 µM (0.5 µCi) [U-^{14}C]proline. Representative of two independent experiments.

A subsequent analysis of proline-derived metabolites by TLC showed four (0.15 mM glucose, Figure 25A) or two (6 mM glucose, Figure 25C) main peaks of labeled metabolites. Retention values were calculated based on standards allowing us to identify labeled proline (peak p, Figure 25) and a second peak co-migrating with alanine and glutamate (peak g/a, Figure 25). Unfortunately, it was not possible to identify peaks x and y (Figure 25). Curiously, in both conditions, there was a reduction in peak y after 5 and 30 minutes labeling with [U-^{14}C]proline (Figure 25) indicating that metabolism of proline might be altered. Analysis of the supernatant of samples incubated in presence of glucose suggested that metabolite y may be excreted and can be detected after 5 min incubation with labeled proline. Since it has been shown before that the excretion products from proline metabolism are mostly glutamate and succinate [145], metabolite y may represent succinate. Glutamate could not be detected in the supernatant during the experiment.

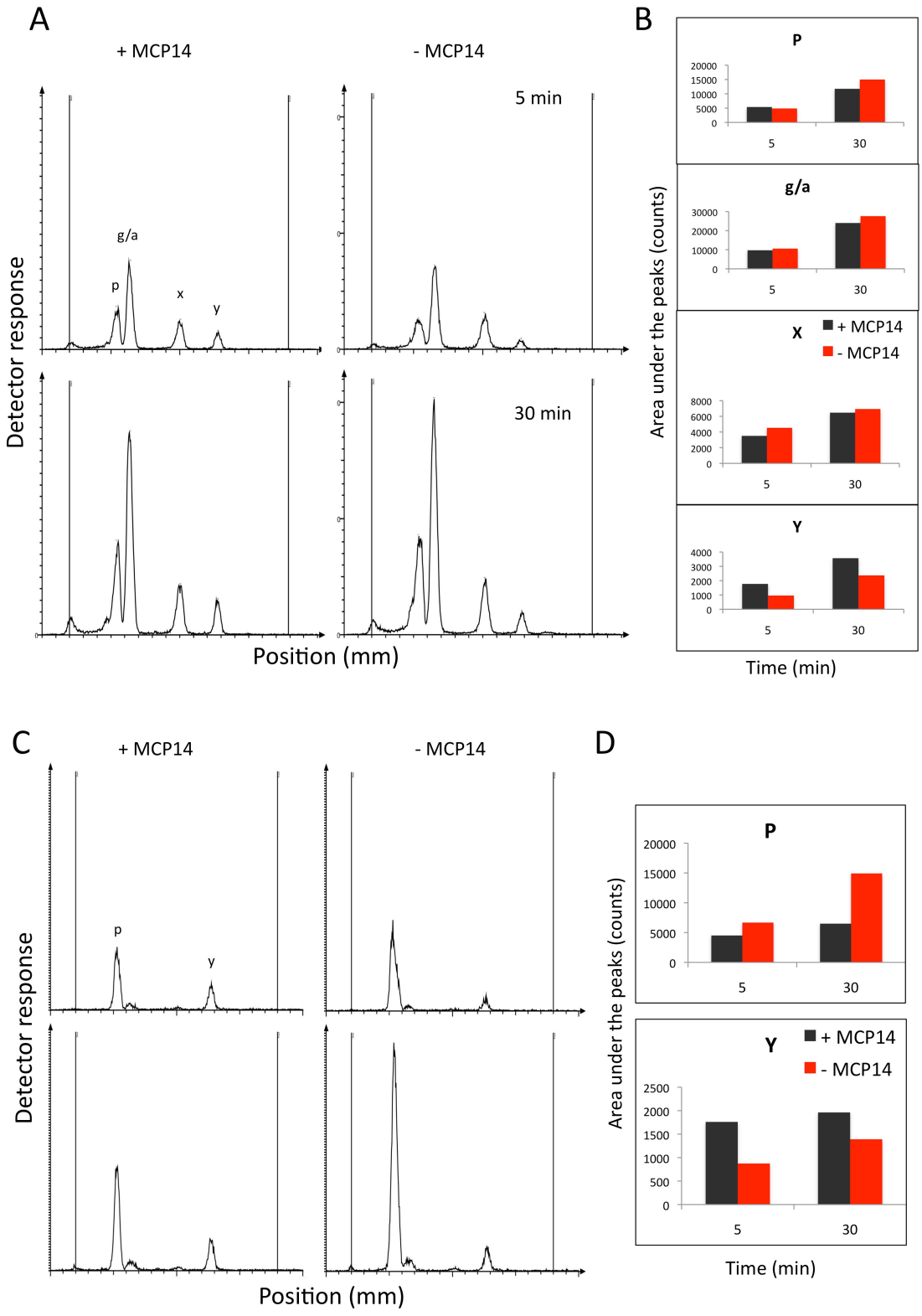


Figure 25. Analysis of proline metabolites in TbMCP14 conditional KO procyclic forms cultivated in glucose-depleted medium (SDM80, A and B) or in presence of glucose (SDM79, C and D). Parasites (5 x 10^6/sample) were incubated in PBS containing 500 µM non-labeled proline and 1 µM (0.25 µCi, A and B) or 2 µM (0.5 µM, C and D) [U-^{14}C]proline. A and C) Analysis by TLC allowed identification of 2 peaks; p, proline, and g/a, glutamate/alanine. Peaks x and y are unknown proline metabolites. B and D) Quantification of each of the peaks showed in A and C, respectively.

3.8 ATP production by digitonin-extracts of TbMCP14 knock-out parasites

Growth curves of TbMCP14 knock-out procyclic forms in glucose-depleted conditions suggested that this mitochondrial carrier could be involved in energy metabolism (Chapter 2.2, Figure 7). To investigate if depletion of TbMCP14 affects ATP production by mitochondria we used digitonin-extracts from TbMCP14 conditional knock-out procyclic forms cultivated in glucose-depleted conditions (SDM80 supplemented with 10% normal FBS → ~0.5 mM of glucose). Growth curve under these conditions was followed and revealed defective growth starting between day 7 and 8 after removing tetracycline (Figure 26A). Subsequently, parasites were permeabilized by digitonin exactly as described in [146] and ATP production was assessed as in [147]. Briefly, the resulting mitochondrial pellet from 10^8 cell equivalents was resupended in 750 µl of import buffer (20 mM Tris-HCl, pH 7.4, 15 mM KH_2PO_4, 0.6 M sorbitol, 10 mM $MgSO_4$, 10 mg/ml fatty-acid-free BSA) and 71.5 µL of suspension was used for each condition. In this assay, ATP production via oxidative phosphorylation is assessed by addition of 5 mM of succinate and 67 µM ADP, whereas ATP production via substrate level phosphorylation can be induced by adding 5 mM 2-ketoglutarate and 67 µM ADP. After incubation at room temperature for 30 min, the reaction was processed, and the ATP concentration was determined as described. Inhibitors were pre-incubated with mitochondria for 10 min on ice and used at the following final concentrations: atractyloside (33.3 μg/ml), malonate (6.7 mM), antimycin (2.7 μM). ATP production was then induced by the addition of substrate and ADP.

Importantly, preservation of an intact outer mitochondrial membrane is crucial to correctly evaluate ATP production via oxidative phosphorylation (advice from André Schneider, University of Bern). Therefore, we followed the recommendation to perform a digitonin titration in order to ensure optimal conditions (not shown). Our preliminary data demonstrated that TbMCP14 may cause a reduction on ATP production by oxidative phosphorylation, while no difference in ATP production was detected when 2-ketoglutarate was added (Figure 26B). Antimycin, specific inhibitor of complex III of respiratory chain, or atractyloside, inhibitor of ADP/ATP translocator, were used to block ATP production. This result suggests reduction on ATP production via oxidative phosphorylation. However, we are still missing consistent data to take conclusions about ATP production by digitonin-extracts of *T. brucei* procyclic forms.

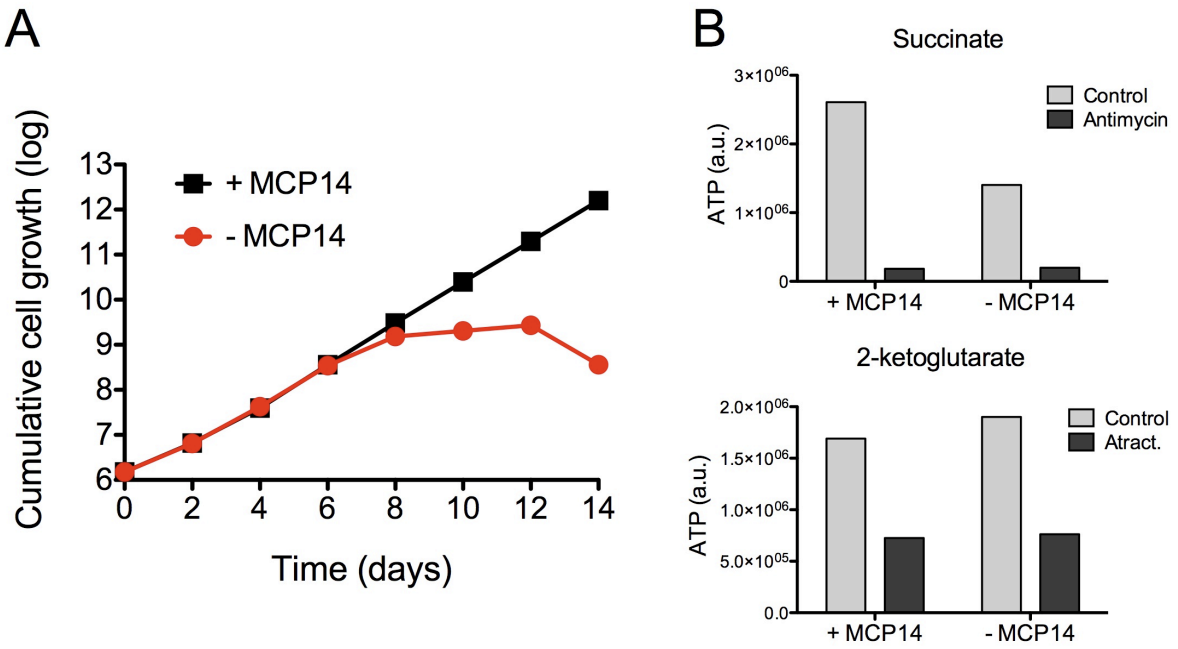


Figure 26. Growth curve (A) and ATP production by digitonin extracts (B) of TbMCP14 conditional knock-out procyclic forms. A) Growth in presence (+ MCP14) or absence (- MCP14) of tertracycline was evaluated in SDM80 without glucose and supplemented with 10% normal (complete) FBS. B) After 6 days, parasites collected and used to perform the digitonin permeabilization. Upper panel, ATP production via oxidative phosphorylation is assessed by addition of ADP and succinate, and can be inhibited by antimycin. Lower panel, ATP production via substrate level phosphorylation can be assessed by addition of ADP and 2-ketoglutarate and is inhibited by atractyloside.

In addition, I have also evaluated the ATP production by isotonically isolated mitochondria [147] from TbMCP14 conditional knock-out. These experiments suggested limited ATP production via oxidative phosphorylation in both conditions (+ and – TbMCP14, not shown), probably due to damaged outer mitochondrial membrane. In contrast, three different experiments done with aliquots of pure mitochondria from the same extract suggested 50% reduction on ATP production via substrate level phosphorylation (not shown). Therefore, these results couldn't be reproduced by using digitonin-extracts so far.

Alternatively, I used the ATP production assays to address whether TbMCP14 could transport glutamate, threonine or fumarate. For that, digitonin-extracts of TbMCP14 conditional knock-outs were used but, unfortunately, production of ATP by addition of glutamate to was comparable in presence or absence of the carrier, whereas not ATP production could be detected when threonine or fumarate were used (not shown). These results indicate that TbMCP14 doesn't work as a glutamate transporter.

3.9 Two additional candidates identified by the RNAi library screen

The library screen described in Chapter 2.2 identified not only TbMCP14, but also additional genes that may be involved in the mode of action of choline analogs (Table 2). The first candidate gene, Tb927.9.3630, was identified in the two independent screens using G25 or T3. The second candidate gene, Tb927.5.3870, was identified in the screen using T4. RNAi constructs of both candidate genes were cloned and transfected into trypanosomes. Unfortunately, I only obtained resistant clones expressing RNAi constructs against Tb927.9.3630 and stopped trying to obtain Tb927.5.3870 RNAi cell lines.

Afterwards, relative quantification of Tb927.9.3630 mRNA by quantitative-PCR suggested ~60% reduction for bloodstream forms, while in procyclic forms down-regulation was not quantified. Growth curves after down-regulation of Tb927.9.3630 in bloodstream forms showed no defect, whereas in procyclic forms, a growth defected could be seen after 6 days of induction with tetracycline (Figure 27). After several attempts, drug-sensitivity assays using these RNAi cell lines after down-regulation of Tb927.9.3630 suggested no difference in sensitivity to G25 or T3 (not shown). However, since Tb927.9.3630 was identified in two independent RNAi library screens, we cannot rule out its involvement in the mode of action of choline analogs. Moreover, proteomic analysis indicated Tb927.9.3630 to be enriched in mitochondrial fraction [148].

Drug	Insert	Annotation	TMD?
G25	Tb927.9.3630	Hypothetical protein	No
	Tb927.10.13120 (MCP14)	Mitochondrial carrier protein	Yes, 5
T3	Tb927.9.3630	Hypothetical protein	No
	Tb927.10.13120 (MCP14)	Mitochondrial carrier protein	Yes, 5
T4	Tb927.5.3870	Hypothetical protein	No
	Tb927.10.13120 (MCP14)	Mitochondrial carrier protein	Yes, 5

Table 2. Genes identified by screening an RNAi library in bloodstream forms [137]. TMD, transmembrane domains.

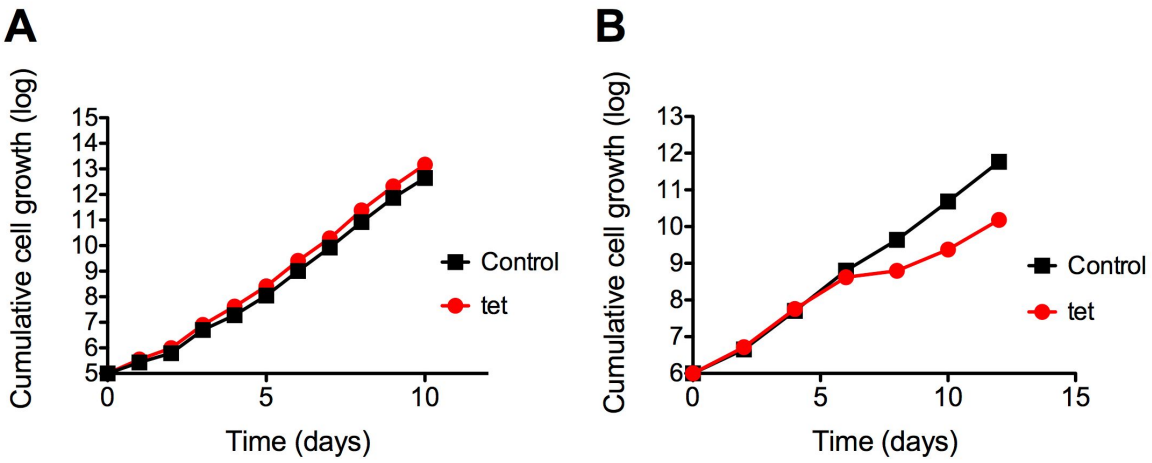


Figure 27. Growth curves of *T. brucei* bloodstream (A) and procyclic (B) forms in absence (control) or in presence (tet) of tetracycline to induce RNAi against Tb927.9.3630.

4. General discussion and perspectives

The present thesis focuses on the mechanism of choline uptake in trypanosomes, a prerequisite for PC synthesis. We studied the inhibition of this process by a group of anti-malarial drugs, known as choline analogs, and in addition, used these drugs to get new insights on choline metabolism and their trypanocidal effect.

We demonstrated for the first time that T. brucei procyclic and bloodstream forms take up choline and readily use it as a substrate for synthesis of PC. This observation has added a possible explanation for the essentiality of the CDP-choline branch of the Kennedy pathway under laboratory conditions; i.e. CDP-choline pathway is essential because synthesis of PC from free choline is a key metabolic pathway in *T. brucei* procyclic and bloodstream forms. Furthermore, we demonstrated for the first time that anti-malarial choline analogs are potent inhibitors of choline uptake in *T. brucei* [85]. We studied the kinetics of this inhibition under culture conditions and demonstrated that the mode action of choline analogs is independent of their potency to inhibit choline uptake [85]. Subsequently, three different choline analogs were used to screen an RNAi library in T. brucei bloodstream forms, resulting in the identification of a mitochondrial carrier, TbMCP14, as key protein in the mode of action of choline analogs. In addition, we demonstrated that the choline analogs target the trypanosome mitochondrion. Together, our findings revealed a previously uncharacterized mitochondrial carrier, TbMCP14, that is directly involved in the mode of action of known trypanocidal drugs.

The mode of action of the drugs in T. brucei differs from the mechanism previously proposed in Plasmodium [100,105,106,107]. This point can be explained partially by two reasons: i) there is no homolog of TbMCP14 in Plasmodium, suggesting that Plasmodium parasites lack a mitochondrial carrier for choline analogs; ii) the fact that *Plasmodium spp.* are intracellular parasites that triggers new permeability pathways to scavenge nutrients and, 'shooting itself in the foot', also promotes uptake of choline analogs, increasing in about 400 times the concentration of these drugs in the parasite's vicinity [103].

Plasmodium spp. are compartmentalized inside erythrocytes, i.e. protected from the host immune system as well as from therapeutic drugs circulating in the plasma. However, it has been observed that T3 accumulates inside Plasmodium-infected erythrocytes compared to non-infected [103], which is consistent with a previous study involving T16, another choline analog [83]. Interestingly, biochemical characterization of T3 transport into infected erythrocytes suggested that

it enters primarily via the so-called new permeability pathway [103]. This pathway is defined as the Plasmodium-induced permeability in red blood cells to low molecular weight substrates allowing the sporozoites to scavenge blood substrates from inside the erythrocyte [149,150]. Thus, toxicity of choline analogs may largely be due this accumulation inside erythrocytes, thereby increasing the concentrations of choline analogs tremendously, leading to higher toxicity to the parasites [103]. Activity of the three enzymes involved in PC synthesis in Plasmodium was investigated in the presence of increasing concentrations of T3 [103]. Interestingly, inhibition was reported only at millimolar concentrations of T3, i.e. at much higher concentrations than those reported to inhibit choline uptake into Plasmodium parasites [103]. Thus, the primary target of T3 is likely the uptake of choline, rather than the inhibition of PC synthesis [103]. For this reason, one of the most promising features of choline analogs in the treatment of malaria is their ability to accumulate inside erythrocytes, by a similar pathway that ensures parasite proliferation, and thus, reducing the risk to develop drug resistance mechanisms.

Is still too early to draw final conclusions about the mode of action of choline analogs in T. brucei, since only two studies have addressed this issue till to date [70,85]. *In vivo* studies will be necessary to evaluate whether choline analogs are suitable compounds in the treatment of sleeping sickness. Based on pharmacodynamics data of T3 [103], we could speculate that trypanosomes are exposed to as little as 100 nM of T3 in the blood. This concentration, based on the cytotoxicity studies involving T3 on parasites under culture conditions (showing an EC_{50} of 1.22 μM), would not affect parasite viability. However, no *in vivo* study using choline analogs to treat trypanosome-infected animals has been done so far. A potentially interesting approach would be to test effectiveness of choline analogs against infections of intracellular trypanosomatids, like *Leishmania* and *T. cruzi*, which have obvious gene homologs of TbMCP14 in their genome. In this case, there would be a possibility to combine the promising feature to accumulate inside the host, delivering high concentrations of drugs, and the potentially high sensitivity of trypanosomatids partially due to presence of TbMCP14 homologs. However, accumulation of choline analogs inside *Leishmania*- or *T. cruzi*-infected cells has never been tested.

A second question raised by our study is related to the functional and essential role of TbMCP14 in *T. brucei*. Our data clearly show that down-regulation or knocking-out of TbMCP14 is lethal for procyclic forms in culture. Interestingly, under glucose-depleted conditions, the defect in growth after depletion of TbMCP14 in procyclic forms occurs earlier compared to parasites cultured in presence of standard concentrations of glucose, suggesting an important role of TbMCP14 in metabolism of amino acids for energy production. Indeed, we obtained different indications of a possible alteration of proline metabolism after changing the expression of TbMCP14; more

specifically, in procyclic forms, over-expression of TbMCP14 caused an accumulation of 1-pyrroline-5-carboxylate (1P5C), a metabolite of proline degradation or synthesis. Furthermore, depletion of TbMCP14 caused changes in proline uptake and metabolism. However, we still don't know how TbMCP14 influences proline metabolism in *T. brucei* procyclic forms. Since it is a mitochondrial carrier, one could hypothesize that TbMCP14 functions as proline carrier, but we don't have direct evidence supporting this hypothesis.

Interestingly, knocking-out TbMCP14 in procyclic forms is also lethal in presence of glucose, suggesting that lethality is not related to lack of ATP, since it is mostly produced by glycolysis under these conditions. Hence, considering our observation that under glucose-depleted conditions, depletion of TbMCP14 results in an earlier growth defect, we speculate that TbMCP14 may play a role in the crosstalk of metabolites between glycolysis and mitochondrial metabolism. It is possible that TbMCP14 transports the end product of glycolysis, pyruvate, which represents a crucial metabolite for production of ATP via substrate level phosphorylation, into mitochondria [151] to produce acetate, an important substrate for fatty acid synthesis [152]. However, preliminary results (not shown) indicated that TbMCP14 knock-out mitochondria are still capable to produce normal levels of ATP in the presence of pyruvate and succinate as subsrates, indicating that it may not transport pyruvate.

Since mitochondrial carriers play a central role in metabolism, finding the physiological role of TbMCP14 could contribute immensely to establish new metabolic pathways in trypanosomes.

5. Acknowledgments

Firstly, I am delighted with the opportunity to achieve this important step, not only for my career, but also for my life. It is hard to find a more challenging and brain-consuming achievement than a doctorate in natural science. It is an intense exercise of critical thinking, dynamic discussions, constructing and transmitting knowledge to people in the field. I am very enthusiastic about the scientific training, and I wish I could still contribute to its constant improvement.

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7. Curriculum vitae and list of publications

JUAN PEREIRA DE MACÊDO
Msc in Biochemestry and Immunology, Biologist.

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1. PERSONAL INFORMATION

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Date of birth: April 16th, 1983
Place fo origin: Belo Horizonte/MG
Nationality: Brazilian

2. DEGREES

April 2010

MSc in Immunology and Biochemistry, Federal University of Minas Gerais, MG, Brazil

March 2009

Teaching license Diploma in Biological Sciences, Federal University of Minas Gerais, MG, Brazil

3. EDUCATION

Since August 2010 - present

PhD thesis in Molecular Biology at Institute of Biochemistry and Molecular Medicine with Prof. Dr. Peter Bütikofer, University of Bern, Switzerland. Thesis: Identification and characterization of putative choline transporters in *Trypanosoma brucei* and involvement of a mitochondrial carrier in drug resistance.

March 2009 – April 2010

Master in Immunology and Biochemistry with Prof. Dr. Leda Quércia Vieira at Biological Sciences Institute, Federal University of Minas Gerais, MG, Brazil.

Master Thesis: The role of superoxide during infection with *Leishmania amazonensis*.

August 2004 – March 2009

Basic studies in Biological Sciences and Teaching, Federal University of Minas Gerais, MG, Brazil

4. PROFESSIONAL EXPERIENCE

August to October 2009

Secondary and high school teacher at Getúlio Vargas State School, Belo Horizonte, MG,

Brazil.

October to December 2008

Trainee in teaching as secondary school teacher at Juscelino Kubistchek de Oliveira State School, Belo Horizonte, MG, Brazil.

February to May 2005

Trainee in teaching as a Primary School Teacher at Colégio Batista Mineiro, Belo Horizonte, MG, Brazil

June to September 2002

Selling promoter at AC3 Merchandising Corporation, Belo Horizonte, MG, Brazil

5. METHODS

Very Good Knowledge of techniques in Immunology and Molecular Biology (ELISA, FACS, Mammalian cells and parasite cultures, Immunofluorescence, DNA/RNA extraction and purification, PCR and Real-time PCR, Northern, Southern and Western blots);

Very good Knowledge and experience with murine model (handling, tissue harvesting, and injections);

Good Knowledge in Biochemistry methods of lipids and metabolites by TLC and transport kinetics (determination of Km and Vmax, competition assays and IC50 determination);

Basic Knowledge in free radical assays (reduction of nitroblue tetrasodium, chemiluminescence, Griess method);

6. PUBLICATIONS IN PEER-REVIEWED JOURNALS

1: **Macêdo JP**, Schmidt RS, Mäser P, Rentsch D, Vial HJ, Sigel E, Bütikofer P. Characterization of choline uptake in Trypanosoma brucei procyclic and bloodstream forms. Mol Biochem Parasitol. 2013 Jul;190(1):16-22.

2: Horta MF, Mendes BP, Roma EH, Noronha FS, **Macêdo JP**, Oliveira LS, Duarte MM, Vieira LQ. Reactive oxygen species and nitric oxide in cutaneous leishmaniasis. J Parasitol Res. 2012;2012:203818.

3: Santiago HC, Gonzalez Lombana CZ, **Macêdo JP**, Utsch L, Tafuri WL, Campagnole-Santos MJ, Alves RO, Alves-Filho JC, Romanha AJ, Cunha FQ, Teixeira MM, Radi R, Vieira LQ. NADPH phagocyte oxidase knockout mice control Trypanosoma cruzi proliferation, but develop circulatory collapse and succumb to infection. PLoS Negl Trop Dis. 2012;6(2):e1492.

4: Gonzalez-Lombana CZ, Santiago HC, **Macêdo JP**, Seixas VA, Russo RC, Tafuri WL, Afonso LC, Vieira LQ. Early infection with Leishmania major restrains pathogenic response to Leishmania amazonensis and parasite growth. Acta Trop. 2008 Apr;106(1):27-38.

8. Declaration of originality

Last name, first name: Pereira de Macêdo, Juan

Matriculation number: 10-121-739

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Bern, 05.05.2014

Signature